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(54) Title
INSECTICIDAL TOXINS, GENES ENCODING THESE TOXINS, ANTIBODIES BINDING TO THEM AND TRANSGENIC PLANT CELLS AND PLANTS EXPRESSING THESE TOXINS

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(57) Claim

1. An insect selective toxin comprising the following amino acid sequence:
VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP GKCR.

2. A recombinant DNA comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

11. A transgenic plant cell comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

FORM 1 SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

APPLICATION FOR A STANDARD PATENT

63127

Ciba-Geigy AG, incorporated in Switzerland, of Klybeckstrasse 141, 4002 Basle, SWITZERLAND, hereby apply for the grant of a standard patent for an invention entitled:

Insecticidal Toxins, Genes Encoding These Toxins, Antibodies Binding to Them and Transgenic Plant Cells and Plants Expressing These Toxins

which is described in the accompanying complete specification.

Details of basic application(s):-

Basic Applic. No:	Country:	Application Date:
285,924	US	19 December 1988
286,002	US	19 December 1988
286,087	US	19 December 1988

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DATED this EIGHTEENTH day of DECEMBER 1989

Ciba-Geigy AG

By: J. Spruson

Registered Patent Attorney

12049 181289

TO: THE COMMISSIONER OF PATENTS
OUR REF: 113538
S&F CODE: 52760

5845/2

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA
PATENTS ACT 1952

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled:

Insecticidal toxins, genes encoding these toxins, antibodies binding to them and transgenic plant cells and plants expressing these toxins

I, Werner Waldegg, c/- Ciba-Geigy AG, Klybeckstrasse 141, 4002 Basle, Switzerland, do solemnly and sincerely declare as follows:-

1. I am authorised by Ciba-Geigy AG, the applicant for the patent to make this declaration on its behalf.

2. The basic applications as defined by Section 141 of the Act were made in United States of America on

   December 19, 1988 (285,924) by Eliahu Zlotkin and Oz Ben-Yehuda;
   December 19, 1988 (286,002) by Eliahu Zlotkin and Michal Eitan and on
   December 19, 1988 (286,087) by Eliahu Zlotkin, Elizabeth Fowler, Rama M. Belagaje and Jean L. Roberts.

3. Eliahu Zlotkin, of Mevobosmat 5, Mevaseret Zion-90805, Israel;
   Michal Eitan, of Kibbutz Ayeleth Hashahar, Upper Galilee, Israel;
   Oz Ben-Yehuda, of 2, Ben Zion St., Jerusalem, Israel;
   Elizabeth Fowler, of 111 Briarcliff Road, Durham, N.C. 27707, U.S.A.
   Rama M. Belagaje, of 7821 Mohawk Lane, Indianapolis, Indiana 46260. U.S.A.
   and Jean L. Roberts, of 203 North Hugo Street, Indianapolis, Indiana 46229, U.S.A., are the actual inventors of the invention and the facts upon which the applicant is entitled to make the application are as follows:

   The applicant is the assignee of the invention from the actual inventors.

4. The basic applications referred to in paragraph 2 of this Declaration were the first applications made in a Convention country in respect of the inventions the subject of the application.

DECLARED at Basle, Switzerland this 1st day of March 1990

CIBA-GEIGY AG

.....
Werner Waldegg

TO: COMMISSIONER OF PATENTS
AUSTRALIA
S&F REF: 113538

S & F Ref: 113538

FORM 10

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

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(ORIGINAL)

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Name and Address of Applicant: Ciba-Geigy AG
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Sydney, New South Wales, 2000, Australia

Complete Specification for the invention entitled:

Insecticidal Toxins, Genes Encoding These Toxins, Antibodies Binding to Them and Transgenic Plant Cells and Plants Expressing These Toxins

The following statement is a full description of this invention, including the best method of performing it known to me/us

5845/3

Case 5-17404/5/6/CGC 1388/9/0

Insecticidal toxins, genes encoding these toxins, antibodies binding to them and transgenic plant cells and plants expressing these toxins

Abstract

This invention relates to transgenic plants, plant cells and microorganisms which by use of recombinant DNA technology have been so transformed that they are capable of synthesizing insect selective toxins from venoms of venomous animals preferably members of the phylum *Arthropoda*, more preferably of members of the class *Arachnida*, most preferably of members of the order *Scorpiones*, and more preferably of members of the class *Chilopoda*. The invention further relates to genes encoding said insect selective toxins and the use of said genes to confer to plants a genetically mediated resistance to insects and to control phytopathogenic insects. The invention also relates to a toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus* with the amino acid sequence VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP GKCR. The invention further relates to antibodies for said toxins and insecticidally effective compositions comprising said toxins.

Case 5-17404/5/6/CGC 1388/P/0

Insecticidal toxins, genes encoding these toxins, antibodies binding to them and transgenic plant cells and plants expressing these toxins

This invention relates to transgenic plants, plant cells and microorganisms which by use of recombinant DNA technology have been so transformed that they are capable of synthesizing insect selective toxins from venoms of venomous animals preferably members of the phylum *Arthropoda*, more preferably of members of the class *Arachnida*, most preferably of members of the order *Scorpiones*, and more preferably of members of the class *Chilopoda*. The invention further relates to genes encoding said insect selective toxins and the use of said genes to confer to plants a genetically mediated resistance to insects and to control phytopathogenic insects. The invention also relates to a toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus*. The invention further relates to antibodies for said toxins and insecticidally effective compositions comprising said toxins.

Venom is defined as a mixture of substances which are produced in specialized glandular tissues in the body of venomous animal. The venom is introduced into the body of its prey or opponent by the aid of a stinging-piercing apparatus in order to paralyze and/or kill it. Scorpions contain in their venom a number of proteins, or neurotoxins, which are toxic and act on the nervous system. The individual neurotoxins differ in their potency on various species of animals.

The venoms derived from scorpions belonging to the *Buthinae* subfamily have three main groups of polypeptide neurotoxins which modify axonal sodium conductance. One group of neurotoxins are the α-toxins, which specifically affect mammals through an extreme prolongation of the action potentials due to a slowing or blockage of the sodium channel in activation (Catterall, 1984; Rochat et al., 1979). The α-toxins AaH1 and AaH2 are found in the venom of the scorpion *Androctonus australis* Hector [from which the first excitatory insect toxin AaIT was isolated (Zlotkin et al., 1971a)]. These α-toxins are unable to produce any effects on blowfly larvae (Zlotkin et al., 1971c). The second group of neurotoxins are the depressant insect selective toxins which induce a progressively developing flaccid paralysis of insects by the blockage of action potentials substantially

due to the suppression of sodium current (Lester et al., 1982; Zlotkin et al., 1985). The third group of neurotoxins are the excitatory insect selective toxins which cause an immediate (knock down) spastic paralysis of insects by the induction of repetitive firing in their motor nerves due to an increase of the sodium peak current and the voltage dependent slowing of its inactivation (Walther et al., 1976; Pelhate and Zlotkin, 1981).

The scorpion venom derived insect toxins are preferably detected and their isolation is monitored by the aid of typical responses on *Sarcophaga* blowfly larva expressed in an immediate and transient contraction paralysis for the excitatory toxins and progressively developing flaccidity for the depressant toxins (Zlotkin et al., 1971b; Lester et al., 1982). In spite of the opposite symptomatology induced by the above depressant and excitatory insect toxins, both affect exclusively sodium conductance and share the same binding site in the insect's neuronal membranes (Zlotkin et al., 1985; Gordon et al., 1984).

Insect-selective toxins have also been identified in venoms from a number of other arthropods (Zlotkin, 1985). The ver oms of braconid wasps are highly toxic to lepidopterous larvae. The venom of the braconid *Bracon hebetor* causes a flaccid paralysis in lepidopterous larvae by inducing presynaptic breakage of the excitatory glutaminergic transmission at the insect neuromuscular junction (Piek et al., 1982). The venoms of solitary wasps are toxic to a large number of insects and spiders from different orders (Rathmeyer, 1962). An example of these venoms is the venom of *Philanthus triangulum* which induces in insects a flaccid paralysis substantially due to presynaptic blockage of neuromuscular transmission; this venom affects both excitatory and inhibitory transmission (May and Piek, 1979). The venom of the black widow spider, *Latrodectus mactans*, contains components which are neurotoxic to insects, but not to mammals and others which specifically affect crustaceens (Fritz et al., 1980; Ornberg et al., 1976).

Venoms produced by centipedes of the genus *Scolopendra* are described by Jangi (1984). Since *Scolopendra* centipedes have limited public health importance, their venoms have, however, not been extensively studied and characterised.

It would be desirable to produce plants which possess a genetically mediated resistance to insects through the introduction of genes which will induce the production of insect selective toxins by the plant tissues. It would further be desirable to obtain such venom and especially to obtain the toxin therein, in a form which is substantially free of natural contaminants. Such purified toxin is useful as an insecticide.

According to a first embodiment of this invention, there is provided an insect selective toxin comprising the following amino acid sequence:

VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP GKCR.

According to a second embodiment of this invention, there is provided a recombinant DNA comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

According to a third embodiment of this invention, there is provided a vector comprising a DNA according to the second embodiment.

According to a fourth embodiment of this invention, there is provided a host organism comprising a vector according to the third embodiment.

According to a fifth embodiment of this invention, there is provided a transgenic plant cell comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

According to a sixth embodiment of this invention, there is provided a transgenic plant and its sexual and asexual progeny comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

According to a seventh embodiment of this invention, there is provided a transgenic microorganism comprising a DNA sequence obtainable from animals encoding an insect selective toxin or a functional derivative or fragment thereof.

According to a eighth embodiment of this invention, there is provided an antibody for an insect selective toxin or a functional derivative or fragment thereof obtainable from members of the order *Scorpiones* or the genus *Scolopendra*.

According to a ninth embodiment of this invention, there is provided an agricultural formulation comprising as active substance an insect selective toxin or a functional derivative or fragment thereof obtainable from animals and one or more agricultural carrier and one or more additional agent with the proviso that the toxin is not dissolved in ethanol plus water, acetone plus water or acetone plus dimethyl sulphoxide.

According to a tenth embodiment of this invention, there is provided a method of controlling phytopathogenic insects comprising the application to the insect or its environment of an insecticidally

effective amount of a transgenic microorganism according to the seventh embodiment, or of a formulation according to the ninth embodiment.

According to an eleventh embodiment of this invention, there is provided a method of protecting crop plants against phytopathogenic insects comprising the transformation of the crop plant with a recombinant DNA according to the second embodiment and further comprising the expression in the plant of an insecticidally effective amount of said insect selective toxin or a functional derivative or fragment thereof.

This invention is directed to insect selective toxins from venoms venomous animals preferably members of the phylum *Arthropoda*, more preferably of members of the class *Arachnida*, most preferably of members of the order *Scorpiones*, and more preferably of members of the class *Chilopoda*, especially the toxin LqhP35 isolated from the venom of a yellow scorpion *L. quinquestriatus hebraeus, Buthinae, Buthidae*, and genes encoding these toxins.

This invention is further directed to the production of transgenic plants which possess a genetically mediated resistance to insects through the introduction of genes which will induce the production of insect selective toxins by plant tissues. This invention further relates to recombinant DNA molecules comprising a genetic sequence coding for a toxin for insects and to insect tolerant, transformed plant cells and transformed plants resulting therefrom. In this invention, the plant cell is transformed by a gene coding for an insect-selective toxin, that upon expression or overexpression, confers insect tolerance.

This invention also relates to plants regenerated from the transformed plant cells and the seed thereof. This invention also relates to progeny of plants regenerated from the transformed plant cells, including mutants and variant progeny as far as they possess the insecticidal or insect tolerant characteristics described.

The present invention comprises a method of protecting plants or plant parts against insect pests. Used in this way, the plant or plant part to be protected is presented with an insecticidally effective amount of one or more toxins, at least one of which is derived from anthropod venom for example scorpion venom.

This method is applicable to the protection of plants or any plant part, including seeds derived from the plants, which are susceptible to insect attack.

This invention also relates to chimeric genetic constructs containing the gene, cloning vectors and hosts, and methods for conferring insect tolerance to plants.

This invention is also directed to the use of these toxins as insecticides. Other toxins, purified e.g. from the venom of the chactoid scorpion, *Scorpio maurus planiatus*, can also be used in this invention.

The invention further concerns antibodies capable of binding to these toxins.

Figures

Fig. 1: Construction of pRK252/Tn903/BglII.

Fig. 2: Construction of pCIB5.

Fig. 3 and 4: Construction of pCIB4.

Fig. 5: Construction of pCIB2.

Fig. 6: Construction of pCIB10, a broad host range plasmid containing T-DNA borders and a gene for plant selection.

Fig. 7: Amino acid sequences of several scorpion toxins determined as described in Example 10. LqhIT2 is the representative toxin of Example 10. LqqIT2 is a depressive insect toxin from *L. quinquestriatus quinquestriatus* whose purification is described in Zlotkin et al. (1985). BjIT2 is a depressive insect toxin from *Buthotus judaicus* whose purification is described by Lester et al. (1982). LqhP35 is described herein and an "intermediate" toxin which affects insect sodium channels in a manner very similar to the effect of α-toxins on mammalian sodium channels. The Smp toxins are purified from the venom of the chactoid scorpion, *Scorpio maurus palmatus*. SmpIT2 is an insect toxin whose purification is described in Lazarovici et al. (1982). SmpCT2 and SmpCT3 are crustacean toxins whose purification is described in Lazarovici et al. (1984). SmpMT is a mammalian toxin whose purification is described in Lazarovici and Zlotkin (1982).

Fig. 8: Synthesis and sequence of the gene for AaIT. Sequence 1a shows the coding strand sequence. Sequence 1b shows the complementary strand sequence. Sequence 1c shows the sequences of the synthesized fragments. Sequence 1d shows the sequence of the final gene.

Fig. 9: Sequence of the gene encoding LqhIT2 insect toxin (designated "Sequence 2" in the Examples).

Abbreviations

AaIT	*Androctonus australis* insect toxin
LqhP35	Lqh represents the scorpion, P indicates paralysis and 35 corresponds to its exclusion time on the HPLC column
HPLC	High Performance Liquid Chromatography

MW	Molecular Weight
SDS-PAGE	Sodiumdodecylsulfate-Polyacrylamide Gelelectrophoresis
PU	Paralytic Unit
3,4-DAP	3,4-diaminopyridine
LD	Lethal Dose
pI	Isoelectric Point
Bp	Base Pairs
CaMV	Cauliflower Mosaic Virus
Asp-N	*Pseudomonas fragi* Endoproteinase Asp-N
Lys-C	Lysin-Endopeptidase
Glu-C	*Staphylococcus aureus* Protease V8
BSA	Bovine Serumalbumin
TTX	Tetrodotoxin
PEG	Polyethyleneglycol
Tris-HCl	Tris(hydroxymethyl)methylamine hydrochloride
EDTA	Ethylenediamine-N,N,N',N'-tetraacetic acid
TFA	Trifluoroacetic acid
4-AP	4-Aminopyridine
NPT	Neomycin-phosphotransferase
STX	Saxitoxin
w/v	Weight/Volume
TEA	Tetraethylammoniumchloride
ATCC	American Type Culture Collection, Rockville, Maryland
D	Dalton

I. Insect Selective Toxins Derived from Venom.

The various genes encoding toxins can be used to transform plants to make them insect tolerant according to this invention.

The amino acid sequence of the first determined insect toxin, an excitatory toxin from *Androctonus australis* (AaIT) was determined and the sequence published in Darbon et al. (1982). The amino acid sequence for this neurotoxin is as follows:
KKNGYAVDSS GKAPECLLSN YCNNQCTKVH YADKGYCCLL SCYCFGLNDD KKVLEISDTR KSYCDTTIIN.

LqqIT2 is a depressive insect toxin from *L. quinquestriatus quinquestriatus* (Zlotkin et al., 1985). The amino acid sequence for this neurotoxin is as follows:

LqqIT2 DGYIRKRDGC KLSCLFGNEG CNKECKSYGG SYGYCWTWGL ACWCEGLPDE KTWKSETNTC G

BjIT2 is a depressive insect toxin from *Buthotus judaicus* (Lester et al., 1982). BjIT2 exists in two isoforms which differ in amino acid sequence at position 15. Form 1 has isoleucine in this position while form 2 has valine. The amino acid sequence for this neurotoxin is as follows:

BjIT2 DGYIRKKDGC KVSC(V/I)IIGNEG CRKECVAHGG SFGYCWTWGL ACWCENLPDA VTWKSSTNTC G

LqhIT2 is a depressive insect toxin from *L. quinquestriatus hebraeus* which is purified using reverse phase HPLC. The amino acid sequence for this neurotoxin is as follows:

LqhIT2 DGYIKRRDGC KVACLIGNEG CDKECKAYGG SYGYCWTWGL ACWCEGLPDD KTWKSETNTC G

SmpIT2, from the chactoid scorpion, *Scorpio maurus palmatus*, is a depressive insect toxin (Lazarovici et al., 1982). The amino acid sequence for this neurotoxin is as follows:

SmpIT2 ALPLSGEYEP CVRPRKCKPG LVCNKQQICV DPK

A new toxin which can be used according to this invention is LqhP35 which induces the delayed and sustained contraction paralysis of blowfly larvae. This toxin thus affects insect sodium channels in a manner very similar to the effect of α-toxins on mammalian sodium channels. This neurotoxin is derived from a yellow scorpion *L. quinquestriatus hebraeus, Buthinae, Buthidae*. The amino acid sequence for this neurotoxin is as follows:

VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP GKCR

When compared to the excitatory and depressant insect selective neurotoxins derived from *Buthinae* scorpion venoms this toxin (a) induces a different symptomatology in blowfly larvae (PU: 14 ng per 100 mg of b.w.) (b) is unable to displace the labeled excitatory insect toxin ^{125}I AaIT from its binding sites in the insect neuronal membrane; (c) possesses a potent toxicity to crustaceans as well (20 ng per 100 mg b.w.) but, however, a very weak toxicity to mice (100 μg per 20 g b.w.) and (d) induces an entirely different effect on sodium conductance in an insect axonal preparation.

Sarcophaga falculata blowfly larvae, due to the segmental arrangement of their skeletal muscles, soft and flexible cuticles and constant mobility, may display simple behavioral responses to different neurotoxins. Such behavioral responses have already enabled the clear distinction among the excitatory and depressant insect toxins derived from *Buthinae* scorpion venoms (Zlotkin, 1986).

In spite of its extreme y low toxicity to mammals, the LqhP35 toxin reveals a strong functional and structural resemblance to the α-toxins affecting mammals derived from *Buthinae* scorpion venoms, and which bind to and probe the voltage sensing or gating structure of sodium channels in vertebrates (Catterall, 1984):

(a) It causes an extreme prolongation of the action potentials in an insect axonal preparation (at about two orders of magnitude lower concentration than the potent α-mammal toxin AaH2) and a rat isolated skeletal muscle fibre (in at least one order of magnitude higher concentration than the AaH2 toxin) due to the slowing of the sodium channel inactivation process.
(b) It reveals about 75 % of amino acid sequence identity with the above α-toxins affecting mammals.

The LqhP35 toxin reveals by its MW, basicity and amino acid composition the typical physicochemical characteristics of the scorpion venom polypeptide neurotoxins (Possani, 1984). Its pharmacology, however, possesses certain unique features. The examination of the pharmacological significance of LqhP35 toxin demands a brief observation of the scorpion toxins, affecting vertebrates, the so-called mammalian toxins. These toxins play an essential role in the pharmacological and chemical characterization of Na^+ channels in mammalian excitable tissues (Catterall, 1984) and are commonly subdivided into two categories: The α-toxins, such as the AaH2 or LqqV, affect sodium inactivation, possess a voltage-dependent binding ability and positive cooperativity with the lipid soluble alkaloids, such as veratridine (Catterall, 1984; Zlotkin et al., 1985).

The β-toxins derived from the venoms of *Centruroides* and *Tityus* scorpions affect sodium activation, possess potential independent binding sites distinct from those of the α-toxins and do not interact synergistically with veratridine (Couraud et al., 1982 and Couraud and Jover, 1984).

The interaction of the insect excitatory toxins (as represented by the AaIT), with the insect neuronal membranes strongly resembles the effect of β-toxins in mammalian neuronal systems, as expressed in the induction of repetitive firing (Pelhate and Zlotkin, 1981) and voltage independent binding (Gordon et al., 1984). With this background it appears now that the LqhP35 toxin reveals an evident α-toxin like action on the insect neuronal membrane.

The resemblance between LqhP35 and the scorpion venom α-toxins has obtained two main expressions - an electrophysiological and a structural one. LqhP35 induces in two different excitable tissue preparations the "classical" effect on the sodium inactivation previously demonstrated by the *Buthinae* scorpion venoms and their derived toxins active to vertebrates (Catterall, 1980). The second resemblance to the α-toxins is expressed in the primary structure of LqhP35. The amino acid sequence of the LqhP35 toxin reveals about 75 % identity with the α-toxins, but only 17 % identity with insect excitatory toxin, respectively. In other words, the similarity of LqhP35 toxin to the α-toxins equals that which occurs among the α-toxins themselves.

Although the LqhP35 toxin has close structural and pharmacological similarities to the α-toxins, the LqhP35 toxin has demonstrated very low toxicity to mammals, in contrast to its relatively high toxicity to insects. The typical α-toxins AaH1 and AaH2 and the β-toxin Css2 are inactive to *Sarcophaga* larvae, and do not reveal any specific binding to an insect neuronal preparation (Zlotkin et al., 1971c; Gordon et al., 1984).

The potent toxicity of the present LqhP35 to arthropods deserves attention since such substances can serve as models for the clarification of the animal group specificity of scorpion venom toxins. The present study, however, is also motivated by an additional consideration, related to the pharmacological value of the α-toxins in the study of the properties and function of the vertebrate sodium channels (Catterall, 1980 and 1984). These toxins may serve as markers and probes of the voltage stimulated conformational change related to the mechanism of the channel opening (Catterall, 1984). The potent toxicity of LqhP35 to insects coupled with its strong effect on the sodium inactivation in an insect axon may be an important pharmacological tool for the study of sodium conductance related to insect neuronal excitability.

The LqhP35 neurotoxin can be utilized as indicated above as a probe for or as a marker of the voltage stimulated conformational change related to the mechanism of the channel

opening in the study of sodium conductance related to insect neuronal excitability. Additionally, the LqhP35 toxin can be used as an insecticide for controlling insect pests.

The venoms from the centipedes, *Scolopendra canidens* and *S. cinqulata* have low toxicity to mice, but a quick and potent effect on insects. In particular, venom from *S. canidens* collected around the Dead Sea has no toxicity to mice at a dose of 1 mg/10 g b.w., but is quite potent on insects. The toxins from centipedes can be used in this invention.

Scolopendra centipedes attack their insect prey (such as, for example, locusts, flies, etc.) using both mechanical and chemical means. Initially, insect prey is trapped by the tight envelopment of the insect by the centipede's powerful legs. After the insect prey has been immobilized, the centipede injects its venom into the insect. The injection of venom induces a rapid paralysis of the prey. The centipede is resistant to its own venom and to the venom of other centipedes of its species. It may resist a dose of venom which would be sufficient to paralyze at least 150 locusts of an equal weight.

Field collected centipedes can be maintained in the laboratory for prolonged periods (of about 1 year). The centipedes are preferably maintained separately in containers having a moisture absorbing substrate, and a source of water. Once every two weeks the centipedes are fed a diet of live insects.

The venom of *Scolopendra* centipedes can most preferably be obtained, for research or other purposes by "milking" the animal's venom glands. Venom milking is preferably accomplished by electrical stimulation at the bases of the venom fangs. The expressed venom is preferably collected into plastic capillary tubes which have been tightly placed on the tip of the fangs.

The volume of venom obtained from such milking is dependent on the dimensions of the centipede, but ranges generally from about 0.25 to about 5 µl. The dry weight of the venom corresponds to approximately 25 %. Approximately 70 % of the dry weight of the venom is protein. The toxicity of the venom in solution is preserved for at least 5 days in room temperature, and it is resistant to lyophilization.

Venom is preferably obtained from any suitable species of centipede. Preferably, venom is obtained from *S. canidens* or *S. cingulata*. *S. canidens*, and especially *S. canidens* collected from the region of the Dead Sea is especially preferred. The invention can,

however, be practiced with other isolates of *S. canidens*, or with other centipedes of the *Scolopendra* genus. Equivalent centipede genera and species may also be employed in accordance with the present invention. The venoms possess the capacity to paralyze locusts when present at concentrations ranging from 0.25 to 6.5 µg venom/g of locust b.w.. The venom of *S. canidens* (Dead Sea) is the most potent to insects and has no effect on mice.

The toxicity of the centipede venom is destroyed by proteolytic enzymes. This finding suggests that the protein nature of the venom is its active component. Separations of centipede venom on a column of MW exclusion in an HPLC system indicate the occurrence of active components in the range of MW of 10 to 130 kD.

It is possible to purify the centipede-derivable insect selective toxin of the present invention using means such as by HPLC, molecular exclusion chromatography, electrophoresis, etc.. For example, the toxin can be fractionated by HPLC and the isolated fractions tested for their capacity to inhibit or kill insects. Fractions having such insecticidal activity can be subjected to additional purification, as desired, until a sample of toxin has been made substantially free of its natural contaminants. Techniques of chromatography are well known in the art and can be readily adapted to the purpose of the present invention by those of ordinary skill.

Alternatively, the toxin molecules can be purified using immunological means, and especially immunoaffinity chromatography.

As used herein, the term "centipede-derivable insect selective toxin" is intended to refer to a chemical toxin which is identical to, or substantially similar to, the insecticidal component of centipede venom. The "centipede-derivable insect selective toxin" of the present invention may be obtained either from a centipede, from peptide or other synthetic chemistry, or by application of the techniques of molecular biology. The toxin is said to be "selective" if it is capable of affecting an insect, but either has no effect, or has a non-substantial effect, on a non-insect.

II. Antibodies to Neurotoxins.

Another aspect of this invention are antibodies to these neurotoxins. In the following description, reference will be made to various methodologies well-known to those skilled

in the art of immunology. Standard reference works setting forth the general principles of immunology include the work of Klein (1982); Kennett et al. (1980); Campbell (1984); and Eisen (1980).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of a hapten which can be recognized and bound by an antibody. An antigen may have one or more than one epitope. An "antigen" is capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The term "antibody" (Ab) or "monoclonal antibody" (Mab) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and $F(ab')_2$ fragments) which are capable of binding an antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl et al., 1983).

To purify an insect selective toxin using antibody affinity chromatography, it is necessary to employ an antibody capable of binding to the toxin. Most preferably, such an antibody will be a monoclonal antibody.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the neurotoxin or a fragment thereof, can be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding the neurotoxin. In a preferred method, a neurotoxin fragment is prepared and purified to render it substantially free of natural contaminants. In another preferred method, a neurotoxin fragment is synthesized, according to means known in the art. Either the purified fragment or the synthesized fragment or a combination of purified natural fragment and/or synthesized fragment may be introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present invention are monoclonal antibodies. Such monoclonal antibodies can be prepared using hybridoma technology (Köhler and Milstein, 1975 and 1976; Köhler et al., 1976; Hämmerling et al., 1981). In

general, such procedures involve immunizing an animal with neurotoxin antigen. The splenocytes of such animals are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP_2O), available from ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium and then cloned by limiting dilution as described by Wands and Zurawski (1981). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the neurotoxin antigen.

If the toxin source is impure, only some of the hybridoma cells will produce antibodies capable of binding to the toxin (other hybridoma cells will produce antibody capable of binding to the toxin contaminants). Thus, it may be necessary to screen among the hybridoma cells for those which are capable of secreting an antibody which is capable of binding to the toxin. Such screening is preferably accomplished by incubating a sample of the toxin (or venom) in the presence of monoclonal antibody secreted from each of a group of particular hybridoma cells and identifying any hybridoma cell capable of secreting an antibody which is able to neutralize or attenuate the ability of the venom to paralyze an insect. Once such a hybridoma cell has been identified, it may be clonally propagated by means known in the art in order to produce the toxin-specific monoclonal antibody.

Once a toxin-specific monoclonal antibody has been obtained, it may be immobilized by binding to a solid support and used to purify the toxin from natural venom or other sources using immunoaffinity chromatography in accordance to methods which are well known in the art. Such methods are capable of mediating a high degree of purification and of thereby producing a toxin which is substantially free of natural contaminants. As used herein, a toxin is said to be "substantially free of natural contaminants" if it is present in a form which lacks compounds with which it is naturally and normally associated (i.e. other proteins, lipids, carbohydrates, etc.).

Once the toxin has been purified, it can be used to immunize an animal (such as a mouse or rabbit) in order to elicit the production of toxin-specific polyclonal antibody.

Thus, one aspect of the present invention concerns such toxin-specific monoclonal and polyclonal antibodies. Another aspect of the present invention concerns a hybridoma cell capable of producing a toxin-specific monoclonal antibody.

Through application of the above-described methods, additional cell lines capable of producing antibodies which recognize epitopes of the desired insect selective toxin can be obtained. Application of the above-described methods is sufficient to permit one to obtain a highly purified toxin preparation.

Through application of the above-described methods, additional cell lines capable of producing antibodies which recognize epitopes of the desired insect selective toxin can be obtained.

III. Genetic Engineering of Insect Selective Toxins.

This invention further comprises the genetic sequences coding for the insect selective toxins, expression vehicles containing the genetic sequence, hosts transformed therewith, the toxin produced by such transformed host expression, and uses for the toxin.

Any of a variety of procedures may be used to clone the toxin-encoding gene sequence. One such method entails analyzing a shuttle vector library of cDNA inserts (derived from a toxin expressing cell) for the presence of an insert which contains the toxin gene sequence. Such an analysis may be conducted by transfecting cells with the vector and then assaying for toxin expression.

One method for cloning the toxin gene sequence entails determining the amino acid sequence of the toxin molecule. To accomplish this task toxin protein may be purified (as described above), and analyzed to determine the amino acid sequence of the proteinaceous toxin. Any method capable of elucidating such a sequence can be employed, however, Edman degradation is preferred. The use of automated sequenators is especially preferred.

The sequence of amino acid residues is designated herein either through the use of their commonly employed single-letter designations. A listing of these one-letter and the three-letter designations may be found in textbooks such as Lehninger (1975). When the amino acid sequence is listed horizontally, the amino terminus is intended to be on the left end whereas the carboxy terminus is intended to be at the right end.

Using the amino acid sequence information, the DNA sequences capable of encoding them are examined in order to clone the gene encoding the toxin. Because the genetic code

is degenerate, more than one codon may be used to encode a particular amino acid (Watson et al., 1977).

Although it is possible to determine the entire amino acid sequence of the toxin, it is preferable to determine the sequence of peptide fragments of the molecule, and to use such sequence data to prepare oligonucleotide probes which can be used to isolate the entire toxin gene sequence. Toxin peptide fragments can be obtained by incubating the intact molecule with cyanogen bromide, or with proteases such as papain, chymotrypsin or trypsin (Oike et al., 1982; Liu et al., 1983).

Using the genetic code (Watson et al., 1977) one or more different oligonucleotides can be identified, each of which would be capable of encoding the toxin peptides. The probability that a particular oligonucleotide will, in fact, constitute the actual toxin encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a particular amino acid) in eukaryotic cells. Such "codon usage rules" are disclosed by Lathe (1985). Using these rules, a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most probable" nucleotide sequence capable of encoding the toxin peptide sequences is identified.

The oligonucleotide, or set of oligonucleotides, containing the theoretical "most probable" sequence capable of encoding the toxin gene fragments is used to identify the sequence of a complementary oligoncleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the toxin gene (Maniatis et al., 1982).

Thus, in summary, the actual identification of toxin peptide sequences permits the identification of a theoretical "most probable" DNA sequence, or a set of such sequences, capable of encoding such a peptide. If the peptides are greater than 10 amino acids long, the sequence information is generally sufficient to permit one to clone a gene sequence such as that encoding the toxin. By constructing an oligonucleotide complementary to this theoretical sequence (or by constructing a set of oligonucleotides complementary to the set of "most probable" oligonucleotides), one obtains a DNA molecule (or set of DNA molecules), capable of functioning as a probe to identify and isolate the toxin gene.

The cloning and use of the various toxins described above will hereinafter be described generally as the cloning and use of "a toxin". It should be understood that any of the above-detailed toxins may be used as described in any of the methods according to this invention. The process for genetically engineering the toxin according to the invention is facilitated through the cloning of genetic sequences which are capable of encoding the toxin and through the expression of such genetic sequences. As used herein, the term "genetic sequences" is intended to refer to a nucleic acid molecule (preferably DNA). Genetic sequences which are capable of encoding the toxin may be derived from a variety of sources. These sources include genomic DNA, cDNA, synthetic DNA, and combinations thereof.

Genomic DNA may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with the 5' promoter region of the toxin gene sequences. To the extent that a host cell can recognize the transcriptional regulatory and translational initiation signals associated with the expression of the protein, then the region 5' may be retained and employed for transcriptional and translational initiation regulation.

For cDNA, the cDNA may be cloned and the resulting clone screened with an appropriate probe for cDNA coding for the desired sequences. Once the desired clone has been isolated, the cDNA may be manipulated in substantially the same manner as the genomic DNA. However, with cDNA there will be no introns or intervening sequences. For this reason, a cDNA molecule which encodes the toxin is the preferred genetic sequence of the present invention.

Genomic DNA or cDNA may be obtained in several ways. Genomic DNA can be extracted and purified from suitable cells by means well known in the art. Alternatively, mRNA can be isolated from a cell which produces the toxin and used to produce cDNA by means well known in the art. Such suitable DNA preparations are enzymatically cleaved, or randomly sheared, and ligated into recombinant vectors to form a gene library. Such vectors can then be screened with the above-described oligonucleotide probes in order to identify a toxin encoding sequence.

A suitable oligonucleotide, or set of oligonucleotides, which is capable of encoding a fragment of the toxin (or which is complementary to such an oligonucleotide, or set of oligonucleotides) identified using the above-described procedure, is synthesized, and

hybridized by means well-known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing the toxin gene. The source of DNA or cDNA used will preferably have been enriched for toxin sequences. Such enrichment can most easily be obtained from cDNA obtained by extracting RNA from cells which produce high levels of the toxin. Techniques of nucleic acid hybridization are disclosed by Maniatis et al. (1982) and by Hames and Higgins (1985).

To facilitate the detection of the desired toxin encoding sequence, the above-described DNA probe may be labeled with a detectable group. Such detectable group can be any material having a detectable physical or chemical property. Such materials have been well-developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (Wisdom, 1976), enzyme substrates (GB 1,548,741), coenzymes (US 4,230,797 and US 4,238,565) and enzyme inhibitors (US 4,134,792); fluorescers (Soini and Hemmilä, 1979); chromophores; luminescers such as chemiluminescers and bioluminescers (Gorus and Schram, 1979); specifically bindable ligands; proximal interacting pairs; and radioisotopes such as ^{3}H, ^{35}S, ^{32}P, ^{125}I and ^{14}C. Such labels and labeling pairs are detected on the basis of their own physical properties (e.g., fluorescers, chromophores and radioisotopes) or their reactive or binding properties (e.g., enzymes, substrates, coenzymes and inhibitors). For example, a cofactor-labeled probe can be detected by adding the enzyme for which the label is a cofactor and a substrate for the enzyme. For example, one can use an enzyme which acts upon a substrate to generate a product with a measurable physical property. Examples of the latter include, but are not limited to, β-galactosidase, alkaline phosphatase and peroxidase.

General procedures for hybridization are disclosed, for example, in Maniatis et al. (1982) and in Hames and Higgins (1985). Those members of the above-described gene sequence library which are found to be capable of such hybridization are then analyzed to determine the extent and nature of the toxin encoding sequences which they contain.

In an alternative way of cloning the toxin gene, a library of expression vectors is prepared by cloning DNA or, more preferably cDNA, from a cell capable of expressing toxin into an expression vector. The library is then screened for members capable of expressing a protein which binds to anti-toxin antibody, and which has a nucleotide sequence that is capable of encoding polypeptides that have the same amino acid sequence as the toxin or fragments of the toxin.

The cloned toxin encoding sequences, obtained through the methods described above, may be operably linked to an expression vector, and introduced into bacterial, or eukaryotic cells to produce toxin, or a functional derivative thereof. Techniques for such manipulations are disclosed by Maniatis et al. (1982) and are well known in the art.

The above discussed methods are, therefore, capable of identifying genetic sequences which are capable of encoding the toxin or fragments thereof. In order to further characterize such genetic sequences, it is desirable to express the toxins which these sequences encode, and confirm that they possess characteristics of toxin peptides. Such characteristics may include the ability to specifically bind anti-toxin antibody, the ability to elicit the production of antibodies which are capable of binding to the toxin, the ability to provide a toxin function to a recipient cell, etc.

In lieu of using the above-described recombinant methods, a gene sequence which encodes the toxin can be prepared by synthetic means (such as by organic synthetic means, etc.).

An alternative way of obtaining a genetic sequence which is capable of encoding the toxin is to prepare it by oligonucleotide synthesis. This method is especially feasible for proteins, such as the toxins of this invention, which have less than 100 amino acids. The genetic code is used to determine an oligonucleotide sequence which is capable of encoding the amino acid sequence.

In a preferred embodiment, this oligonucleotide sequence is predicted using the codon frequency appropriate for the organism in which the gene is to be expressed. Such codon frequencies for some organisms are available as part of the sequence analysis computer programs of the University of Wisconsin Genetics Computer Group. Codon frequencies for other organisms may be calculated with the aid of the same computer package using data in the available sequence data banks. In some cases, alternative codons may be selected to facilitate synthesis and/or provide convenient restriction sites. Translational stop and start signals are added at the appropriate points and sequences to create convenient cloning sites are added to the ends. The above nucleotide sequence constitutes the "coding strand". The sequence of the "complementary strand" is predicted using the computer programs mentioned above.

A series of oligonucleotides ranging from 20 to 50 bases is synthesized in order to provide a series of overlapping fragments which when annealed and ligated will produce both strands of the gene. These fragments are then annealed and ligated together using techniques well known to those skilled in the art (Maniatis et al., 1982). The resulting DNA fragment with the predicted size is isolated by electrophoresis and ligated into a suitable cloning vector for amplification and further manipulation. This synthetic gene may be handled using the techniques described above for genes isolated from genomic and/or cDNA.

IV. Expression of the Insect Selective Toxin and its Functional Derivatives.

The toxin encoding sequences, obtained through the methods described above, may be operably linked to an expression vector, and introduced into prokaryotic or eukaryotic cells in order to produce the toxin or its functional derivatives. The present invention pertains both to the intact toxin and to the functional derivatives of this toxin.

As used herein, the term "substantially pure" or "substantially purified" is meant to describe the neurotoxin which is substantially free of any compound normally associated with the toxin in its natural state, i.e., free of protein and carbohydrate components. The term is further meant to describe the neurotoxin which is homogeneous by one or more purity or homogeneity characteristics used by those of skill in the art. For example, a substantially pure neurotoxin will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: MW, chromatographic behaviour, and such other parameters. The term, however, is not meant to exclude artificial or synthetic mixtures of the neurotoxin with other compounds. The term is also not meant to exclude the presence of minor impurities which do not interfere with the biological activity of the neurotoxin and which may be present, for example, due to incomplete purification.

Also, as used herein, "LqhP35," "the LqhP35 neurotoxin," and "the LqhP35 toxin" are used interchangeably and as exemplification to denote the toxin derived from the scorpion venom. The present invention pertains both to the intact neurotoxin and to the functional derivatives of the LqhP35 toxin.

A "functional derivative" of the toxin is a compound which possesses a biological activity (either functional or structural) that is substantially similar to a biological activity of the

toxin. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule. A "fragment" of a molecule such as the toxin is meant to refer to any polypeptide subset of the molecule. A "variant" of a molecule such as the toxin is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof. A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if the structure of one of the molecules is not found in the other, or if the sequence of amino acid residues is not identical. An "analog" of a molecule such as the toxin is meant to refer to a molecule substantially similar in function to either the entire molecule or to a fragment thereof. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. Moieties capable of mediating such effects are disclosed in Osol, A. (Ed.) Remington's Pharmaceutical Sciences (Mack Publishing Co., Easton, PA, 1980). Procedures for coupling such moieties to a molecule are well known in the art.

A DNA sequence encoding the toxin or its functional derivatives may be recombined with vector DNA in accordance with conventional techniques, including use of blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Maniatis et al. (1982) and are well known in the art.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and if such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of the toxin synthesis. Such regions will

normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the gene sequence coding for the toxin may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the toxin, the transcriptional termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and the toxin encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the toxin gene sequence, or (3) interfere with the ability of the toxin gene sequence to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

Thus, to express the toxin transcriptional and translational signals recognized by an appropriate host are necessary.

The present invention also encompasses the expression of the toxin protein (or a functional derivative thereof) in either prokaryotic or eukaryotic cells. Preferred prokaryotic hosts include bacteria such as *E. coli*, *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, etc.. The most preferred prokaryotic host is *E. coli*. Bacterial hosts of particular interest include *E. coli* K12 strain 294 (ATCC 31446), *E. coli* X1776 (ATCC 31537), *E. coli* W3110 (F^-, λ^-, prototrophic (ATCC 27325)), and other enterobacteria such as *Salmonella typhimurium* or *Serratia marcescens*, and various *Pseudomonas* species. Under such conditions, the toxin will not be glycosylated. The procaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

To express the toxin (or a functional derivative thereof) in a prokaryotic cell (such as, for example, *E. coli*, *B. subtilis*, *Pseudomonas*, *Streptomyces*, etc.), it is necessary to operably link the toxin encoding sequence to a functional prokaryotic promoter. Such promoters

may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive promoters include the *int* promoter of bacteriophage λ, the *bla* promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325, etc.. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage λ (P_L and P_R), the *trp*, *recA*, *lacZ*, *lacI*, and *gal* promoters of *E. coli*, the α-amylase promotor (Ulmanen et al., 1985) and the σ-28-specific promoters of *B. subtilis* (Gilman et al., 1984), the promoters of the bacteriophages of *Bacillus* (Gryczan, 1982), and *Streptomyces* promoters (Ward et al., 1986). Prokaryotic promoters are reviewed by Glick and Whitney (1987); Cenatiempo (1986); and Gottesman (1984).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene-encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold et al. (1981).

Preferred eukaryotic hosts include yeast, fungi, insect cells, mammalian cells either in vivo, or in tissue culture. Mammalian cells which may be useful as hosts include cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/O-AG14 or the myeloma P3x63Sg8, and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as well as neuroblastoma cell lines such as IMR 332 that may provide better capacities for correct post-translational processing.

For a mammalian host, several possible vector systems are available for the expression of the toxin. A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or signals which are subject to chemical regulation, e.g., by metabolite.

Yeast provides substantial advantages in that it can also carry out post-translational peptide modifications. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes produced in large quantities when yeast are grown in media rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene sequence can be utilized.

Another preferred host is insect cells, for example the *Drosophila* larvae. Using insect cells as hosts, the *Drosophila* alcohol dehydrogenase promoter can be used (Rubin, 1988). Alternatively, baculovirus vectors can be engineered to express large amounts of the toxin in insects cells (Jasny, 1987; Miller et al., 1986).

As discussed above, expression of the toxin in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer and Walling, 1982); the TK promoter of Herpes virus (McKnight, 1982); the SV40 early promoter (Benoist and Chambon, 1981); and the yeast *gal4* gene promoter (Johnston and Hopper, 1982; Silver et al., 1984).

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the toxin (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in the formation of a fusion protein (if the AUG codon is in the same reading frame as the toxin encoding DNA sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the toxin encoding sequence).

The toxin encoding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a non-replicating DNA (or RNA) molecule which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the toxin may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome.

In one embodiment, a vector is employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama and Berg (1983).

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184, πVX). Such plasmids are, for example, disclosed by Maniatis et al. (1982). *Bacillus* plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan (1982). Suitable *Streptomyces* plasmids include pIJ101 (Kendall and Cohen, 1987), and *Streptomyces* bacteriophages such as Φ2C31 (Chater et al., 1986). *Pseudomonas* plasmids are reviewed by John and Twitty (1986) and Isaki (1978).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-μ circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein et al., 1982; Broach, 1981 and 1982; Bollon and Stauver, 1980; Maniatis, 1980).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the toxin, or in the production of a fragment of this toxin. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like).

The expressed protein may be isolated and purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like.

V. Uses of the Insect Selective Toxins to Genetically Modify Plants.

The genes encoding the insect selective toxins according to the present invention can be introduced into a plant by genetic engineering techniques, which upon production of the toxins in the plant cell could be used as a means for controlling insect pests. Therefore, it is possible to produce a plant that is more insect-tolerant. In thus another embodiment of this invention, the toxin gene is used to transform a plant to enhance the insect tolerance of the plant.

The coding region for a toxin gene that may be used in this invention may be the full-length or partial active length of the gene. It is necessary, however, that the genetic sequence coding for the toxin be expressed, and produced, as a functional toxin in the resulting plant cell.

DNA from both genomic DNA and cDNA and synthetic DNA encoding a toxin may be used in this invention. Further, a toxin gene may be constructed partially of a cDNA clone, partially of a genomic clone, and partially of a synthetic gene and various combinations

thereof. In addition, the DNA coding for the toxin gene may comprise portions from various species.

There are a variety of embodiments encompassed in the broad concept of the invention. In one embodiment of this invention, the insect selective neurotoxin may be combined with another compound or compounds to produce unexpected, such as synergistic properties. These other compounds can include protease inhibitors, for example, which have oral toxicity to insects. Other compounds that could be used in combination with the insect selective toxin to genetically modify a plant to confer insect tolerance include polypeptides from *Bacillus thuringiensis*. The *B. thuringiensis* protein causes changes in potassium permeability of the insect gut cell membrane (Sacchi et al., 1986) and is postulated to generate small pores in the membrane (Knowles and Ellar, 1987). Other pore-forming proteins could also be used in combination with the toxins. Examples of such pore-forming proteins are the magainins (Zasloff, 1987), the cecropins (Hultmark et al., 1982), the attacins (Hultmark et al., 1983), melittin, gramicidin S (Katsu et al., 1988), sodium channel proteins and synthetic fragments (Oiki et al., 1988), the α-toxin of *Staphylococcus aureus* (Tobkes et al., 1985), apolipoproteins and their fragments (Knott et al., 1985; Nakagawa et al., 1985), alamethicin and a variety of synthetic amphipathic peptides (see review by Kaiser and Kezdy, 1987). Lectins (Lis and Sharon, 1986) which bind to cell membranes and enhance endocytosis are another class of proteins which could be used in combination with insect-selective toxins to genetically modify plants for insect resistance.

In another embodiment, this invention comprises chimeric genetic sequences:

(a) a first genetic sequence coding for a toxin that upon expression of the gene in a given plant cell is functional for the toxin and optionally a second genetic sequence coding for a polypeptide having oral toxicity to insects;
(b) one or more additional genetic sequences operably linked on either side of the toxin coding region. These additional genetic sequences contain sequences for promoter(s) or terminator(s). The regulatory sequences may be heterologous or homologous to the host cell.

In a preferred embodiment, the promoter of the toxin gene is used to express the chimeric genetic sequence. Other promoters that may be used in the genetic sequence include *nos*, *ocs*, and CaMV promoters. An efficient plant promoter that may be used is an overproducing promoter. This promoter in operable linkage with the genetic sequence for

the toxin should be capable of promoting expression of said toxin such that the transformed plant has increased tolerance to insect pests. Overproducing plant promoters that may be used in this invention include the promoter of the gene encoding the small subunit (ss) of the ribulose-1,5-bisphosphate carboxylase from soybean (Berry-Lowe et al., 1982), and the promoter of the gene encoding the chlorophyll a/b binding protein. These two promoters are known to be light induced in plant cells (see, for example, Cashmore, 1983; Corruzi et al., 1983; and Dunsmuir et al., 1983).

Further, in another preferred embodiment, the expression of the chimeric genetic sequence comprising the toxin gene is operably linked in correct reading frame with a plant promoter and with a secretion signal sequence.

The chimeric genetic sequence comprising a toxin gene operably linked to a plant promoter, and in the preferred embodiment with the secretion signal sequences, can be ligated into a suitable cloning vector. In general, plasmid or viral (bacteriophage) vectors containing replication and control sequences derived from species compatible with the host cell are used. The cloning vector will typically carry a replication origin, as well as specific genes that are capable of providing phenotypic selection markers in transformed host cells, typically resistance to antibiotics. The transforming vectors can be selected by these phenotypic markers after transformation in a host cell.

Host cells that may be used in this invention include procaryotes, including bacterial hosts such as *E. coli*, *Salmonella typhimurium*, and *Serratia marcescens*. Eucaryotic hosts such as yeast or filamentous fungi may also be used in this invention.

The cloning vector and host cell transformed with the vector are used in this invention typically to increase the copy number of the vector. With an increased copy number, the vectors containing the toxin gene can be isolated and, for example, used to introduce the chimeric genetic sequences into the plant or other host cells.

Plant tissue is transformed with the vectors described above by any technique known in the art. Such methods used for transfer of DNA into plant cells include, for example, the direct infection of or co-cultivation of plants, plant tissue or cells with *A. tumefaciens* (Horsch et al., 1985; Marton, 1984), direct gene transfer of exogenous DNA to protoplasts (Paszkowski et al., 1984; EP 129,668; EP 164 575; Shillito et al., 1985; Potrykus et al., 1985; Lörz et al., 1985; Fromm et al., 1985 and 1986; GB 2,140,822; and Negrutiu et al.,

1987); incubation with PEG (Negrutiu et al., 1987); micro-injection (Reich et al., 1986a and b), and microprojectile bombardment (Klein et al., 1987).

Another method of introducing the toxin gene into plant cells is to infect a plant cell with *A. tumefaciens* transformed with the toxin gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots, roots, and develop further into transformed plants. The toxin genetic sequences can be introduced into appropriate plant cells, for example, by means of the Ti plasmid of *A. tumefaciens*. The Ti plasmid is transmitted to plant cells on infection by *A. tumefaciens* and is stably integrated into the plant genome (Horsch et al., 1984; Fraley et al., 1983).

Ti plasmids contain two regions essential for the production of transformed cells. One of these, named transfer DNA (T DNA), induces tumor formation. The other, termed virulent region, is essential for the formation but not maintenance of tumors. The T DNA region, which transfers to the plant genome, can be increased in size by the insertion of an enzyme's genetic sequence without its transferring ability being affected. By removing the tumor-causing genes so that they no longer interfere, the modified Ti plasmid can then be used as a vector for the transfer of the gene constructs of the invention into an appropriate plant cell.

All plant cells which can be transformed by *Agrobacterium* and whole plants regenerated from the transformed cells can also be transformed according to the invention so to produce transformed whole plants which contain the transferred toxin gene.

There are presently two different ways to transform plant cells with *Agrobacterium*:

(1) co-cultivation of *Agrobacterium* with cultured isolated protoplasts, or
(2) transforming cells or tissues with *Agrobacterium*.

Method (1) requires an established culture system that allows culturing protoplasts and plant regeneration from cultured protoplasts.

Method (2) requires (a) that the plant cells or tissues can be transformed by *Agrobacterium* and (b) that the transformed cells or tissues can be induced to regenerate into whole plants. In the binary system, to have infection, two plasmids are needed: a T DNA containing plasmid and a *vir* plasmid.

In an alternative embodiment of this invention, the toxin gene may be introduced into the plant cells by electroporation (Fromm et al., 1985). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the toxin genetic construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus. Selection of the transformed plant cells with the expressed toxin can be accomplished using the phenotypic markers as described above.

The exogenous DNA may be added to the protoplasts in any form such as, for example, naked linear, circular or supercoiled DNA, DNA encapsulated in liposomes, DNA in spheroplasts, DNA in other plant protoplasts, DNA complexed with salts, and the like.

The genetic material may also be transferred into the plant cell by using PEG which forms a precipitation complex with the genetic material that is taken up by the cell (Paszkowski et al., 1984).

Transfer of DNA into plant cells is also achieved by injection into isolated protoplasts, cultured cells and tissues (Reich et al., 1986a and b) and injection into meristematic tissues of seedlings and plants (de La Peña et al., 1987; Graves and Goldman, 1986; Hooykaas-Van Slogteren et al., 1984; and Grimsley et al., 1987 and 1988). Transgenic plants and progeny therefrom are obtained by conventional methods known in the art.

Another method to introduce foreign DNA sequences into plant cells comprises the attachment of said DNA to particles which are then forced into plant cells by means of a shooting device as described by Klein et al. (1988). Any plant tissue or plant organ may be used as the target for this procedure, including but not limited to embryos, apical and other meristems, buds, somatic and sexual tissues in vivo and in vitro. Transgenic cells and callus are selected following established procedures. Targeted tissues are induced to form somatic embryos or regenerate shoots to give transgenic plants according to established procedures known in the art. The appropriate procedure may be chosen in accordance with the plant species used.

The regenerated plant may be chimeric with respect to the incorporated foreign DNA. If the cells containing the foreign DNA develop into either micro- or macrospores, the integrated foreign DNA will be transmitted to sexual progeny. If the cells containing the foreign DNA are somatic cells of the plant, non-chimeric transgenic plants are produced

by conventional methods of vegetative propagation either in vivo, from buds or stem cuttings, or in vitro following established procedures known in the art. Such procedures may be chosen in accordance with the plant species used.

After transformation of the plant cell or plant, those plant cells or plants transformed so that the enzyme is expressed, can be selected by an appropriate phenotypic marker. These phenotypic markers include, but are not limited to, antibiotic resistance. Other phenotypic markers are known in the art and may be used in this invention.

Due to the variety of different transformation systems all plant types can in principle be transformed so that they express a toxin of the present invention thus being resistent to insects. Prefered, however, are those methods using transformation with *Agrobacterium*, and especially those using direct gene transfer. In the case of direct gene transfer those methods are prefered where protoplasts are transformed.

All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are recovered which contain the transferred toxin gene. Some suitable plants include, for example, species from the genera *Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Dactylis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hemerocallis, Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browallia, Glycine, Lolium, Zea, Triticum, Sorghum* and *Datura.*

There is an increasing body of evidence that practically all plants can be regenerated from cultured cells or tissues, including but not limited to all major cereal crop species, sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables. Limited knowledge presently exists on whether all of these plants can be transformed by *Agrobacterium.* Species which are a natural plant host for *Agrobacterium* may be transformable in vitro. Monocotyledonous plants, and in particular, cereals and grasses, are not natural hosts to *Agrobacterium.* Attempts to transform them using *Agrobacterium* have been unsuccessful until recently (Hooykas-Van Slogteren et al., 1984). There is growing evidence now that certain monocots can be transformed by *Agrobacterium.* Using novel experimental

approaches that have now become available, cereal and grass species may be transformable.

Additional plant genera that may be transformed by *Agrobacterium* include *Ipomoea, Passiflora, Cyclamen, Malus, Prunus, Rosa, Rubus, Populus, Santalum, Allium, Lilium, Narcissus, Ananas, Arachis, Phaseolus*, and *Pisum*.

Plant regeneration from cultured protoplasts is described in e.g. Evans and Bravo (1983); Davey (1983); Dale (1983); and Binding (1985).

Regeneration varies from species to species of plants, but generally a suspension of transformed protoplasts containing multiple copies of the toxin gene is first provided. Embryo formation can then be induced from the protoplast suspensions, to the stage of ripening and germination as natural embryos. The culture media will generally contain various amino acids and hormones, such as auxins and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

The mature plants, grown from the transformed plant cells, are selfed to produce an inbred plant. The inbred plant produces seed containing the gene for the toxin. These seeds can be grown to produce plants that have the toxin.

The inbreds according to this invention can e. g. be used to develop insect tolerant hybrids. In this method, an insect tolerant inbred line is crossed with another inbred line to produce the hybrid.

Parts obtained from the regenerated plant, as flowers, seeds, leaves, branches, fruit, and the like are covered by the invention provided that these parts comprise the insect tolerant cells. Progeny and variants, and mutants of the regenerated plants are also included within the scope of this invention.

In diploid plants, typically one parent may be transformed by the toxin genetic sequence and the other parent is the wild type. After crossing the parents, the first generation hybrids (F_1) will show a distribution of 1/2 toxin/wild type : 1/2 toxin/wild type. These

first generation hybrids (F_1) are selfed to produce second generation hybrids (F_2). The genetic distribution of the F_2 hybrids is 1/4 toxin/toxin : 1/2 toxin/wild type : 1/4 wild type/wild type. The F_2 hybrids with the genetic makeup of toxin/toxin are chosen as the insect tolerant plants.

As used herein, variant describes phenotypic changes that are stable and heritable, including heritable variation that is sexually transmitted to progeny of plants, provided that the variant still comprises an insect tolerant plant. Also, as used herein, mutant describes variation as a result of environmental conditions, such as radiation, or as a result of genetic variation in which a trait is transmitted meiotically according to well-established laws of inheritance. The mutant plant, however, must still exhibit an insect tolerance according to the invention.

VI. Use of the Insect-Selective Toxins to Improve Insecticidal Microbes

The insect selective toxin alone or in combination with any of the enhancing compounds mentioned-above may be used to enhance the toxicity of insecticidal microbes. Several baculoviruses including those that infect *Heliothis virescens* (cotton bollworm), *Orgyia pseudotsugata* (Douglas fir tussock moth), *Lymantia dispar* (gypsy moth), *Autographica californica* (alfalfa looper), *Neodiprion sertifer* (European pine fly), and *Laspeyresia pomonella* (codling moth) have been registered and used as pesticides. Introduction of an insect-selective toxin into the genome could significantly enhance the potency of such pesticides. Methods for the introduction of foreign genes into the genome of baculoviruses are the subject of two patents (US 4,745,051 and EP 175 852). EP 225 777 discloses the production of a microbial insecticide effective against two species of insects by construction of a recombinant baculovirus containing DNA segments of two species of nuclear polyhedrosis (baculovirus) virus. Numerous fungi are capable of infecting insects. Introduction of the insect-selective toxin into the genome of such fungi could enhance the potency as pesticides. *Beauvaria bassania* and *B.brongniartii* have a wide host range and have been suggested as candidates for microbial pesticides (see review by Miller et al., 1983). Bacteria (other than *B. thuringiensis*) that have been considered as insect control agents include *B. popilliae, B. lentimorbus* and *B. sphaericus*. Their potential as pesticides could be enhanced by improving their potency by incorporating an insect-selective toxin gene into their genome.

VII. Application of the toxins as insecticides on plants

The presentation of an insecticidally effective amount of one or more toxins may be achieved by external application. This application of the toxin to the plants or plant parts may be either directly or in the vicinity of the plants or plant parts.

The natural toxin and/or its recombinant equivalent may be applied in a wide variety of forms including powders, crystals, suspensions, emulsified suspensions, dusts, pellets, granules, encapsulations, microencapsulations, aerosols, solutions, gels or other dispersions. This invention therefore also provides a composition for application to plants comprising one or more toxins together with additional agents.

Compositions embraced by this invention generally are applied to the plant or plant part in an agricultural formulation which comprises one or more agricultural carrier. An agricultural carrier is a substance which may be used to dissolve, disperse or diffuse an active compound in the composition without impairing the biological effectiveness of the compound. Such a carrier by itself has no detrimental effect on the soil, equipment, crops or agronomic environment. The composition of this invention may be either solid or liquid formulations or solutions. The compound may be formulated as wettable powders, or a concentrate which is emulsifiable.

Additional agents may be included as adjuvants, such would be wetting agents, spreading agents, dispersing agents, or adhesives, according to conventional agricultural practices.

An additional kind of adjuvants would include potentiators of activity of the toxin. Such potentiators would include lectins, amphipathic peptides, amphipathic proteins or protease inhibitors.

Adjuvants for the formulation of insekticides are well-known to those skilled in the art.

Thus, preferred embodiments of the present invention include but are not restricted to:

An insect selective toxin comprising the following amino acid sequence:
VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA LPDNVPIRVP GKCR.

A recombinant DNA comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

A recombinant DNA comprising a DNA sequence wherein said DNA sequence is obtainable from animals preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra* and encodes an insect selective toxin or a functional derivative or fragment thereof wherein said DNA sequence is in an expressible form.

A recombinant DNA comprising a DNA sequence obtainable from animals wherein said DNA sequence is in an expressible form and encodes an insect selective toxin with the following amino acid sequence:
KKNGYAVDSS GKAPECLLSN YCNNQCTKVH YADKGYCCLL SCYCFGLNDD KKVLEISDTR KSYCDTTIIN,
DGYIRKRDGC KLSCLFGNEG CNKECKSYGG SYGYCWTWGL ACWCEGLPDE KTWKSETNTC G,
DGYIRKKDGC KVSC(V/I)IIGNEG CRKECVAHGG SFGYCWTWGL ACWCENLPDA VTWKSSTNTC G,
DGYIKRRDGC KVACLIGNEG CDKECKAYGG SYGYCWTWGL ACWCEGLPDD KTWKSETNTC G,
ALPLSGEYEP CVRPRKCKPG LVCNKQQICV DPK or
VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG K GNACWCYA LPDNVPIRVP GKCR or a functional derivative or fragment thereof.

A vector comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is in an expressible form.

A host organism comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is in an expressible form.

A transgenic plant cell comprising a DNA sequence obtainable from animals preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra*

wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

A transgenic plant cell comprising a DNA sequence obtainable from animals preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra* wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is stably integrated into the plant genome.

A transgenic plant cell comprising a DNA sequence obtainable from animals preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra* wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is in an expressible form.

A transgenic plant cell that expresses an insect selective toxin encoded by said DNA sequence, or a functional derivative or fragment thereof.

A transgenic plant and its sexual and asexuell progeny comprising a DNA sequence obtainable from animals preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra* wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

A transgenic plant and its sexual and asexuell progeny comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is stably incorporated in the plant genome.

A transgenic plant and its sexual and asexuell progeny comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is in an expressible form.

A transgenic plant and its sexual and asexuell progeny that expresses the insect selective toxin encoded by said DNA sequence, or a functional derivative or fragment thereof.

A transgenic microorganism comprising a DNA sequence obtainable from animals

preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra* wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

A transgenic microorganism comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is stably incorporated in the genome.

A transgenic microorganism comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and is in an expressible form.

A transgenic microorganism expressing the insect selective toxin encoded by said DNA sequence, or a functional derivative or fragment thereof.

An antibody for an insect selective toxin or a functional derivative or fragment thereof obtainable from members of the order *Scorpiones* or the genus *Scolopendra*.

An insecticidal composition comprising as active substance an insect selective preferably recombinant toxin or a functional derivative or fragment thereof obtainable from animals.

A method of controlling phytopathogenic insects comprising the application to the insect or its environment of an insecticidal amount of a transgenic microorganism comprising a DNA sequence obtainable from animals preferably from arthropods, more preferably from members of the classes *Arachnida* or *Chilopoda*, most preferably from members of the order *Scorpiones* and the genus *Scolopendra* wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof wherein said DNA sequence is in an expressible form and is stably incorporated in the genome, or of a composition comprising as active substance an insect selective preferably recombinant toxin or a functional derivative or fragment thereof obtainable from animals.

A method of protecting crop plants against phytopathogenic insects comprising the transformation of the crop plant with a recombinant DNA wherein the recombinant DNA comprises a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof and further

comprising the expression in the plant of an insecticidally effective amount of said insect selective toxin or a functional derivative or fragment thereof.

Having now generally described this invention, the same will be better understood by reference to specific examples, which are included herein for purposes of illustration only, and are not intended to be limiting unless otherwise specified.

EXAMPLES

Example 1: Purification, Primary Structure and Mode of Action of LqhP35

The crude venom of the scorpion *L. quinquestriatus hebraeus* is obtained by an electrical milking (Zlotkin and Gordon, 1985) of field collected scorpions followed by subsequent lyophilization. The excitatory insect toxin AaIT derived from the venom of the scorpion *Androctonus australis* is purified according to Zlotkin et al. (1971a). The mammal α-toxin AaH2 is obtained from Prof. H. Rochat (Fac. Medicine, Biochemie, Marseille, France).

Larvae of the blowfly *Sarcophaga falculata* (100 to 130 mg b.w.) are bred in the laboratory according to Zlotkin et al. (1971b). Isopods (terrestrial crustacean) *Hemilepistus sp.* (300 to 400 mg b.w.) are field collected and albino laboratory mice (variant "Sabra") are purchased from the laboratory animal farm of the Hadassah Medical School, Jerusalem.

Locust synaptosomes and their derived membrane vesicles are prepared from homogenates of the dissected central nervous systems of *Locusta migratoria*, according to a previously described method (Zlotkin and Gordon, 1985).

The lethal and paralytic effects of toxic substances are determined by subcutaneous injections. Blowfly larvae, injected with volumes of 2 to 10 µl per 100 mg of b.w. into an intersegmental membrane of an abdominal segment, are employed in an assay representing a new symptomatology expressed as a delayed and sustained spastic paralysis. The determination of the paralytic unit (PU) is based on immobility accompanied by the contraction of the animal 5 min after injection. Isopods, dorsally injected between the thorax and the abdomen with volumes of 1 to 5 µl per 300 mg of b.w., are used for the determination of the PU based on a complete immobility 5 min after injection. Mice are used for the determination of the fifty percent lethal dose (LD_{50}) as monitored 24 hr after

the injection. The sampling and the calculation of 50 % end points (PU and LD_{50} doses) are based on Reed and Muench (1938).

In the present study three different methods of column chromatography are employed:

a) Molecular exclusion columns of Sephadex G50 fine (Pharmacia, Sweden) gel with the ammonium acetate volatile buffer: 1650 A_{280} units *L. quinquestriatus hebraeus* venom (which correspond to 2.0 g of the crude venom following water extraction and two steps of Sephadex G50 column chromatography) are repeatedly charged on a series of four columns [4x(100x3.2 cm)] equilibrated and eluted (45 ml/hr) by 0.1 M ammonium acetate pH 8.5 buffer (Zlotkin et al., 1971a). The various fractions are collected according to the elution profile. Fraction IV (A_{280} = 200 units) possesses paralytic potency and lethality to blowflies, isopods, and mice.

b) Cation exchange chromatography on the carboxy methyl cellulose (CM52, Whatman, England) resin with a molarity gradient elution with ammonium acetate buffer: 23 mg (31.5 A_{280} units of Fr. IV, a) are charged on a column of 10 ml volume filled with cation exchanger CM-cellulose-CM52 (Whatman, England) equilibrated and eluted (10 ml/hr) by ammonium acetate 0.01 M pH 6.4 buffer. The first stage of elution is performed in equilibrium conditions resulting in fractions (a) and (b) which induce flaccidity to *Sarcophaga* larvae. The second stage of elution is performed in a linear gradient of molarity from 0.1 to 0.5 M resulting in fractions (c), (d) and (e), the toxicity of which to fly larvae and mice is indicated in Table 1.

c) HPLC reversed phase chromatography employing a TSK-RP-CL8 column (LKB, Sweden): Buffers: A: 0.1 % TFA; B: 0.1 % TFA, acetonitrile : isopropanol = 1 : 1. B gradient: 0 time 5 %; 15 min 20 %; 75 min 50 %. Flow rate 1 ml/min.

Slab gel electrophoresis is employed in two forms:

a) SDS-PAGE in the presence of urea (Swank and Munkres, 1971): The separations are performed on a continuous minigel (60x80x1.5 mm) in a concentration of 12.5 % polyacrylamide in the presence of 8 M urea.

b) Analytical isoelectric focusing in polyacryl- amide in the presence of ampholines (LKB, technical bulletin 1217-2001ME).

Protein is determined by the procedure of Lowry et al. (1951) using BSA as standard.

LqhP35 toxin is reduced and alkylated with 4-vinylpyridine by incubating samples in 6 M guanidine HCl, 1 M Tris-HCl, pH 8.6, 10 mM EDTA, 20 mM dithiothreitol for 1 hr at 37°C. 4-Vinylpyridine (Sigma, USA) is added to 50 mM and incubation continued for 1 hr at room temperature. The modified protein is desalted by HPLC on a Vydac-C-8 or Hypersil-ODS column in 0.1 % isopropanol : acetonitrile = 1:1. Peptides are prepared by digestion of the reduced and alkylated protein with Asp-N, Lys-C and Trypsin (Boehringer-Mannheim, USA) according to the manufacturers directions. Peptides are produced by partial acid hydrolysis. Peptides are separated by HPLC on a Hypersil-ODS column in 0.1 % TFA using a gradient of 0 to 60 % isopropanol : acetonitrile = 1:1. The amino acid sequence analysis is performed by the automated Edman degradation with an Applied biosystems 470A gas-phase sequencer (USA). Phenylthiohydantoin amino acids are identified using the on-line Applied Biosystems 120APTH Analyzer. Each sequence is confirmed in at least two separate determinations [Allen, 1981; Inglis, 1980 (Inglis, A. et al., in Birr, C. (Ed.), Methods in Peptide and Protein Sequence Analysis, Proc. Int. Conf. 3rd., Elsevier, Amsterdam, 329, 1980)]. For sequence comparisons amino acid sequences are aligned for maximum homology with the aid of the University of Wisconsin Genetics Computing Group Profile Analysis (Devereux et al., 1984). The percent of total positions containing identical residues is calculated.

^{125}I-labeled AaIT and the competitive displacability binding assays are prepared and performed according to a previously described procedure (Zlotkin and Gordon, 1985).

Insect axonal preparation. Voltage clamp and current clamp experiments are performed on giant axons dissected from abdominal nerve cords of the cockroach *Periplaneta americana* using the double oil-gap, single-fibre technique (Pichon and Boistel, 1967). Normal physiological saline has the following composition: 200 mM NaCl; 3.1 mM KCl; 5.4 mM $CaCl_2$; 5.0 mM $MgCl_2$. The pH is maintained at 7.2 using a phosphate-carbonate buffer (2 mM $NaHCO_3$, 0.1 mM NaH_2PO_4). Current clamp experiments are performed at 20 to 22°C, voltage clamp experiments are performed at 12 ± 0.5°C.

4-AP (concentrations indicated in the text) is employed in order to selectively block the potassium current (Pelhate and Pichon, 1974) and 2×10^{-7} M synthetic STX is used for selective, reversible blockage of sodium currents (Sattelle et al., 1979). The purified scorpion toxins are lyophilized in the presence of BSA (Fraction V, Armour Co., USA) in the ratio of 1/10 (w/v).

Mammalian skeletal muscle single fibre preparation. Voltage-clamp and current-clamp experiments are performed at room temperature (18 to 22°C) on single muscle fibers isolated from the slow twitch soleus muscle of the rat *Rattus norvegicus* using the double mannitol gap single fibre technique (Duval and Léoty, 1978). Normal physiological saline has the following composition: 140 mM NaCl; 6 mM KCl; 3 mM $CaCl_2$; 5 mM glucose. pH 7.3 is adjusted by Tris-HCl (6.5 mM). 10 mM TEA and 2 mM 3,4 DAP are added to the bath in order to block potassium current and 1 µM TTX is used in order to block the sodium current (Duval and Léoty, 1980).

B. Isolation of a factor inducing a delayed and sustained contraction of blowfly larvae. The lyophilized crude venom of the scorpion *L. quinquestriatus hebraeus* (3.7 g) is obtained using the following preliminary treatments: (1) Water extraction and lyophilization; (2) The lyophilized water extract is separated on a column of Sephadex G50 eluted by 0.1 M acetic acid in order to remove high MW mucoproteins; (3) The lyophilized toxic fractions obtained from the above Sephadex-acetic acid column are separated on a column of Sephadex G50 eluted by ammonium acetate 0.1 M, pH 8.5 buffer, in order to remove non protein low MW pigments. The lyophilized toxic fractions obtained in the above step (3) are separated by the recycling method on a series of four Sephadex G50 columns in the conditions specified above. This approach results in the separation of four main fractions (I to IV). Fraction IV (which corresponds to about 12% of the proteins charged) when injected to fly larvae induces a quite uncommon mixture of symptoms including flaccidity (typical to the depressant insect toxins) and also contractivity (typical to the excitatory insect toxins) which, however, occurs after a delay and has a prolonged duration. Fraction IV is also moderately lethal to mice (LD_{50}: 50 µg per 20 g b.w.). This lethality is accompanied by excitatory symptoms of envenomation typical of *Buthinae* scorpion venoms and their derived mammal toxins (Rochat et al., 1979).

The separation of the above Sephadex G50 fraction IV on a column of a cation exchanger (CM52) results in a series of fractions (a) to (e). Fractions (a) and (b) induce the flaccid paralysis of blowfly larvae typical of the depressant insect toxins (Zlotkin, 1986). Fractions (c), (d), and (e), however, demonstrate toxicity to mice (Table 1) and a clear new symptomatology to blowfly larvae expressed in the occurrence of delayed and sustained (of long duration) contraction paralysis. As shown in Table 1, fractions (c), (d) and (e) reveal various degrees of the above delayed-sustained contraction paralysis and mice lethality. Fraction (d), which has the highest toxicity to blowfly and the lowest toxicity to

mice is subjected to further purification.

Table 1: The Toxicity to Blowfly Larvae and Mice of the Fractions (c), (d), and (e) Obtained by CM52 Chromotography

The Assay	The Fraction c	d	e
Blowfly larvae-PU_{50}[a] (µg/100 mg b.w.)	0.054	0.028	0.7
Mice lethality-LD_{50}[b]	12.0	120.0	25.0

[a]An immobile and contracted larva 5 min after injection is considered as a positive response.

[b]Lethality is determined after 24 hr. The LD_{50} of the fraction IV to mice corresponds to 40 µg/20 g b.w.

The final purification of the new factor affecting blowfly larvae is achieved by an additional step of chromatography on a reverse phase column in an HPLC system. The final product is designated as LqhP35 toxin (Lqh represents the scorpion, P indicates paralysis and 35 corresponds to its exclusion time on the HPLC column). The resulting product possesses about 30 and 60 percent of CM52 fraction (d) protein content and activity to blowfly larvae, respectively. Its purity and properties are assessed by SDS-PAGE (indicating a MW of about 5 kD) and by analytical isoelectric focusing (indicating a pI of about pH 9.0).

C. Primary structure determination of the LqhP35 toxin. LqhP35 is a single chained protein composed of 64 amino acids of a MW of about 7 kD (MW = 7255) typical of various scorpion venom toxins (Possani, 1984). The present MW estimation is essentially in accordance with the above SDS-PAGE which yields only approximate data with an expected inaccuracy of at least 20 % (Swank and Munkres, 1971). The high isoelectric point (pI) obtained by analytical isoelectric focusing assay is essentially in accordance with sequence analysis which reveals an excess of positively charged residues over the negatively charged including the occurrence of the three arginines (pK = 12.48). The hydrophobic amino acids, which comprise a third of the residues, are evenly distributed throughout the length of the molecules. It is believed that the eight cysteines form four

disulphide bridges. This aspect is indirectly indicated by the pI value (pH 9.0 to 9.2) of the LqhP35 toxin. At the above pH value, if the sulfhydryl groups were free, they would contribute negative charges due to their ionization, thus lowering the pI value. The occurrence of a cysteine-arginine originating at the C-terminal as in the LqhP35 toxin, has been shown already in the LqqIV mammalian α-toxin derived from the venom of the related scorpion *L. quinquestriatus quinquestriatus* (Possani, 1984).

D. Biological activity of the LqhP35 toxin. Symptomatology. In contrast to the excitatory insect toxins which induce an immediate and transient contraction paralysis of blowfly larvae, the LqhP35 toxin induces a delayed and sustained contraction paralysis.

Toxicity. The paralytic and lethal potency of the LqhP35 toxin to arthropods and mice respectively is presented in Table 2.

Table 2: Toxic Activity of LqhP35 Toxin

Test Animal	The Effect	ED_{50} Value
Blowfly larvae	Delayed sustained contraction paralysis (PU)	14 ng/100 mg b.w.
Isopods (terrestrial crustaceans)	Paralysis within 5 minutes (PU)	20 ng/100 mg b.w.
Mice	Lethality observed after 24 hr (LD_{50})	100 µg/20 g b.w.[a]

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[a]About two orders of magnitude less toxic than the common mammal toxins derived from scorpion venom (Rochat et al., 1979).

Binding assays. 210 µl of reaction mixture includes 1.5 nM of the ^{125}I AaIT, 40 µg of protein in the form of locust synaptosomal membrane vesicles (Zlotkin and Gordon, 1985) and increasing concentrations of the competing substance in the standard binding medium [0.15 M choline chloride; 1 mM $MgSO_4$; 2 mM $CaCl_2$; 0.1 % BSA (Zlotkin and Gordon, 1985)]. The membranes are incubated for 40 min at 22°C. The separation between the free

and the membrane bound ^{125}I AaIT is performed by a rapid filtration method (Zlotkin and Gordon, 1985). The binding of the labeled toxin measured in the presence of a large excess of unlabeled toxin (1 µM) is defined as the non-specific binding.

In contrast to the excitatory (Zlotkin et al., 1985; Gordon et al., 1984) and depressant (LqqIT2, Zlotkin et al., 1985) toxins, the LqhP35 toxin is unable to displace the ^{125}I AaIT toxin in a locust synaptosomal preparation. This may indicate that the LqhP35 toxin possesses distinct binding sites, which differ from those shared by the above excitatory and depressant insect toxins.

Electrophysiological studies. The LqhP35 toxin is assayed in current and voltage clamp conditions in two different preparations of excitable membrane: the isolated giant axon of *Periplaneta americana* and the isolated skeletal muscle fiber of the rat. The effect of the LqhP35 toxin on the action potentials of the cockroach axon is determined by evoking a short (0.5 msec) depolarizing current pulse of 10 nA. The action of the LqhP35 toxin on the Na^+ current of the cockroach axon is determined in a voltage clamp experiment in the presence of 2×10^{-4} M 3,4-DAP. The effect of the LqhP35 toxin on the action potentials in isolated rat soleus muscle fibers is determined by the comparison of action potentials in normal ringer and after external application of the toxin. The effects of LqhP35 on ionic currents of the voltage clamped rat soleus muscle fiber is determined in a voltage clamp experiment (stepwise depolarization from a holding potential of -90 mV to -40 mV).

In both preparations the toxin induces essentially the same effect, the prolongation of the action potential due to an evident inhibition of sodium inactivation. The toxin does not affect or modify the (a) amplitude of the action potentials; (b) the level of the membrane resting potential and (c) potassium conductance.

The LqhP35 toxin reveals an obvious preference for the insect excitable membrane when compared by its activity to the potent mammal toxin AaH2, which is assayed on the same preparations. In the current clamp conditions the prolongation of the action potential is caused by LqhP35 at a concentration about two orders of magnitude lower than that required for the AaH2 toxin in the insect axonal preparation (Pelhate and Zlotkin, 1981) and at least one order of magnitude higher than AaH2 in the rat skeletal muscle membrane. In the latter, however, the maximal duration of the action potential is evidently lower with LqhP35 toxin (10^{-6} M, 2234 ± 584 msec, n = 7) than that with AaH2 (10^{-7} M, 700 ± 420 msec, n = 8).

Example 2: Collection of the centipede venom

S. canidens are isolated from the Dead Sea region and from the Jerusalem region. Field collected centipedes are separately maintained in a laboratory in containers having a moisture absorbing substrate, and a source of water. Centipedes are fed living insects (once in two weeks).

Venom is withdrawn from the centipedes by milking in the following manner: the bases of the venom fangs are electrically stimulated, and venom is collected into plastic capillary tubes which are tightly placed on the tip of the fangs.

Table 3 shows the body lengths of the centipedes, the volume of venom obtained per milking, and the protein concentration of the isolated venom.

Table 3: Centipede Venom Volume and Protein Contents

The Centipede	Body length (cm)	Volume of venom per milking (µl)	Protein content[a] (µg/µl)
S. canidens (Dead Sea region)	6 - 8	0.29, 0.2-0.4 (5)	210
S. canidens Jerusalem region)	11 - 14	4.33, 3.7-50 (3)	190

[a]According to Lowry et al. (1951).

Example 3: Stability of Centipede Venom

The centipede venom from *S. canidens* from the Dead Sea region is isolated as described above, and tested for its stability upon storage at room temperature or after lyophilization. The same batch of *S. canidens* (Dead Sea region) venom as tested in Example 2 is employed. The results of this experiment are presented in Table 4.

Table 4: Stability of Centipede[a] Venom

Conditions[b]	Freshly milked venom	Storage of 5d at room temp.	Deep freezing and lyophilization
Activity[c]	2.6	2.5	31

[a]*S. canidens* from Dead Sea area.
[b]In all the treatments venom is diluted by bidistilled water.
[c]Activity is determined as a paralytic unit of *Sarcophaga* fly larvae expressed in ng of protein (Lowry et al., 1951) per 100 mg of b.w.

Example 4: Toxicity of *Scolopendra* Centipedes Venom to Various Animals

Venoms are collected from three species of centipedes: *S. canidens* (Dead Sea region); *S. canidens* (Jerusalem region) and *S. cingulata* (collected at the Upper Galilee and Golan heights). The toxicity of the lyophilized venoms to three insect species and to mice is presented in Table 5. As shown the venom of *S. canidens* from the Dead Sea region possesses the highest toxicity to insects and is, in practice, inactive to mice. Thus, the toxin in this venom is insect selective. The injection of 1 mg per 10 g mouse does not induce even symptoms of envenomation.

Table 5: Toxicity of *Scolopendra* Centipedes Venom to Various Animals

Centipede Assay[a]	*S. cingulata* (µg)	*S. canidens* (from Jerusalem) (µg)	*S. canidens* (from Dead Sea) (µg)
Paralysis of *Sarcophaga* fly larvae	0.13	0.14	0.005
Lethality to *Sarcophaga* fly larvae	0.075	0.10	0.04
Paralysis of *Spodoptera* larvae	8.23	8.82	2.99
Lethality of *Spodoptera* larvae	8.23	8.82	2.99
Paralysis of *Locusta* adults	0.65	0.64	0.031
Lethality of *Locusta* adults	0.16	0.15	0.043
LD_{50} of mice[b]	350	245	> 1000

[a]Paralysis is determined as PU_{50} expressed in mg per 100 mg of b.w.. Paralysis is determined after 30 sec for *Sarcophaga* larvae and 5 minutes for the *Spodoptera* and *Locusta* insects. Lethality is determined after 24 hr as LD_{50} expressed in µg per 100 mg of b.w.. The sampling (5 or 7 animals per dose) and calculation of the efficient doses - 50%, are performed according to Reed and Muench (1938). The average b.w. of the various animals are: 130 to 150 mg, 70 to 400 mg, 1.3 to 1.6 g and 7 to 12 g for *Sarcophaga* larvae, *Spodoptera* larvae, *Locusta* male adults and albino mice, respectively.

[b] Expressed in µg per 10 g of b.w.

Example 5: Response of a Centipede to the Venom From the Same Species

S. canidens from the Dead Sea region is resistant to the venom of its own species and that it may resist a dose of venom which may paralyze at least 150 locusts of an equal weight (Table 6).

Table 6: Response of a Centipede[a] to the Venom From the Same Species

Centipede	Weight (mg)	Venom injected		Effect	
		(µg prot.)[b]	Locust paralytic units[c]	Immediate	After 24 hrs
1	420	2.2	25	No effect	No effect
2	460	7.4	75	No effect	No effect
3	490	10.5	100	No effect	No effect
4	580	18.7	150	No effect	No effect
5	580	18.7	150	Transient[d]	No effect paralysis
6	580	18.7	150	Transient[d]	Dead paralysis

[a]*S. canidens* - collected at the Dead Sea region.

[b]Determined according to Lowry et al. (1951).

[c]The paralytic potency of the venom sample to *Locusta migratoria* is 21.5 ng per 100 mg of b.w. The number of locust paralytic units, corresponds to locusts of a b.w. identical to that of the respective centipedes.

[d]The paralysis is localized around the injection site and disappears after 20 min.

Example 6: Loss of Toxicity by Heating

The effect of heat (80°C, 5 min) on venom stability is determined using *Sarcophaga* fly larvae. As shown in Table 7, the toxicity of the centipede venom is destroyed by test treatment as determined according to paralytic affect to *Sarcophaga* larvae.

Table 7: Loss of Toxicity by Heating[a]

Venom source	Untreated 2 µl (2 PU_{50})	Heated (80°C, 5 min) 10 µl (10 PU_{50})
S. canidens Dead Sea	Active	Not active
S. canidens Jerusalem	Active	Not active

[a]The PU_{50} for 100 mg b.w. of *Sarcophaga* fly larvae are 5 ng and 150 ng of the Dead sea and Jerusalem centipedes, respectively.

Example 7: The effect of proteolytic enzymes on the toxicity of centipede venom to *Sarcophaga* larvae

The toxicity of centipede venom is destroyed by common proteolytic enzyme preparations (Table 8). Trypsin appears to be more effective than pronase. This result indicates that the centipede toxin is a protein.

Table 8: The Effect of Trypsin and Pronase E (Sigma USA) in a 5% Enzyme/Substrate Ratio on the Toxicity of *S. canidens* (Jerusalem) venom to *Sarcophaga* larvae[a]

Incubation time (hr)	Untreated 2 PU_{50} injected	Trypsin 10 PU_{50} injected	Pronase E 10 PU_{50} injected	Trypsin injected	Pronase injected
1	+	-	+	-	-
5	+	-	-	-	-

[a]The medium is phosphate buffered saline (pH 7.4 - Sigma USA). The PU_{50} of the venom is 150 ng/100 mg b.w.

Example 8: Purification of centipede venoms

Centipede venoms are partially purified by fractionation using an analytical HPLC molecular exclusion column and by reverse phase chromatography.

Analysis using the molecular exclusion column (Suprose 12 10/30 Pharmacia; 0.05 M ammonium acetate pH 8.5; flow rate of 0.5 ml/min) indicates that only about 20 % of the toxicity to fly larvae is recovered from the venom of the Dead Sea centipede. A qualitative distinction is observed between factors inducing flaccidity and those which are contractive to fly larvae. In the Dead Sea centipede the latter correspond to factors of an estimated MW of 15 to 20 kD.

Reversed Phase HPLC analysis employs C-8 (Merck); A (water + 0.1 % TFA); B (isopropanol + acetonitrile + 0.1 % TFA).

The elution patterns of the three venoms are in disaccordance to the "official" taxonomic definitions of the centipede species. The *S. canidens* of Jerusalem and *S. cingulata* demonstrate identical elution patterns on the molecular exclusion and reverse phase chromatographies - both differing from that of the Dead Sea (*S. canidens*) centipede. With this background it is noteworthy that the three species are easily distinguishable according to their dimensions and patterns of coloration.

Example 9: Construction of a Ti plasmid-derived vector

The vector pCIB10 (Rothstein et al., 1987) is a Ti-plasmid-derived vector useful for transfer of the chimeric gene to plants via *A. tumefaciens*. The vector is derived from the broad host range plasmid pRK252, which may be obtained from Dr. W. Barnes, Washington University, St. Louis, Mo. The vector also contains a gene for kanamycin resistance in *Agrobacterium*, from Tn903, and left and right T-DNA border sequences from the Ti plasmid pTiT37. Between the border sequences are the polylinker region from the plasmid pUC18 and a chimeric gene that confers kanamycin resistance in plants.

First, plasmid pRK252 is modified to replace the gene conferring tetracycline-resistance with one conferring resistance to kanamycin from the transposon Tn903 (Oka et al., 1981), and is also modified by replacing the unique EcoRI site in pRK252 with a BglII site (Fig. 1 summarizes these modifications). Plasmid pRK252 is first digested with endonucleases

SalI and SmaI, then treated with the large fragment of DNA polymerase I to create flush ends, and the large vector fragment purified by agarose gel electrophoresis. Next, plasmid p368 which contains Tn903 on an approximately 1050 bp BamHI fragment is digested with endonuclease BamHI, treated with the large fragment of DNA polymerase, and an approximately 1050 bp fragment is isolated after agarose gel electrophoresis; this fragment contains the gene from transposon Tn903 which confers resistance to the antibiotic kanamycin (Oka et al., 1981). Plasmid p368 has been deposited with ATCC, accession number 67700. Both fragments are then treated with the large fragment of DNA polymerase to create flush ends. Both fragments are mixed and incubated with T4 DNA ligase overnight at 50°C. After transformation into *E. coli* strain HB101 and selection for kanamycin resistant colonies, plasmid pRK252/Tn903 is obtained.

Plasmid pRK252/Tn903 is digested at its unique EcoRI site, followed by treatment with the large fragment of *E. coli* DNA polymerase to create flush ends. This fragment is added to synthetic BglII restriction site linkers, and incubated overnight with T4 DNA ligase. The resulting DNA is digested with an excess of BglII restriction endonuclease and the larger vector fragment purified by agarose gel electrophoresis. The resulting fragment is again incubated with T4 DNA ligase to recircularize the fragment via its newly added BglII cohesive ends. Following transformation into *E. coli* strain HB101, plasmid pRK252/Tn903/BglII is obtained (Fig. 1).

A derivative of plasmid pBR322 is constructed which contains the Ti plasmid T-DNA borders, the polylinker region of plasmid pUC19, and the selectable gene for kanamycin resistance in plants (Fig. 2). Plasmid pBR325/Eco29 contains the 1 5 kbp EcoRI fragment from the nopaline Ti plasmid pTiT37. This fragment contains the T-DNA left border sequence (Yadav et al., 1982). To replace the EcoRI ends of this fragment with HindIII ends, plasmid pBR325/Eco29 DNA is digested with EcoRI, then incubated with nuclease S1, followed by incubation with the large fragment of DNA polymerase to create flush ends, then mixed with synthetic HindIII linkers and incubated with T4 DNA ligase. The resulting DNA is digested with endonucleases ClaI and an excess of HindIII, and the resulting 1.1 kbp fragment containing the T-DNA left border is purified by gel electrophoresis. Next, the polylinker region of plasmid pUC19 is isolated by digestion of the plasmid DNA with endonucleases EcoRI and HindIII and the smaller fragment (approx. 53 bp) is isolated by agarose gel electrophoresis. Next, plasmid pBR322 is digested with endonucleases EcoRI and ClaI, mixed with the other two isolated fragments, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid,

pCIB5, contains the polylinker and T-DNA left border in a derivative of plasmid pBR322 (Fig. 2).

A plasmid containing the gene for expression of kanamycin resistance in plants is constructed (Fig. 3 and 4). Plasmid Bin 6 (Bevan, 1984) is obtained from Dr. M. Bevan, Plant Breeding Institute, Cambridge, UK. Plasmid Bin 6 DNA is digested with EcoRI and HindIII and the fragment approximately 1.5 kbp in size containing the chimeric NPT gene is isolated and purified following agarose gel electrophoresis. This fragment is then mixed with plasmid pUC18 DNA which has been cleaved with endonucleases EcoRI and HindIII. Following incubation with T4 DNA ligase, the resulting DNA is transformed into *E. coli* strain HB101. The resulting plasmid is called pUC18/neo. This plasmid DNA contains an unwanted BamHI recognition sequence between the NPT gene and the terminator sequence of the nopaline synthase gene (Bevan, 1984). To remove this recognition sequence, plasmid pUC18/neo is digested with endonuclease BamHI, followed by treatment with the large fragment of DNA polymerase to create flush ends. The fragment is then incubated with T4 DNA ligase to recircularize the fragment, and is transformed into *E. coli* strain HB101. The resulting plasmid, pUC18/neo (Bam) has lost the BamHI recognition sequence.

The T-DNA right border sequence is then added next to the chimeric NPT gene (Fig. 4). Plasmid pBR325/Hind23 contains the 3.4 kbp HindIII fragment of plasmid pTiT37. This fragment contains the right T-DNA border sequence (Bevan et al., 1983). Plasmid pBR325/Hind23 DNA is cleaved with endonucleases SacII and HindIII and a 1.0 kbp fragment containing the right border is isolated and purified following agarose gel electro-phoresis. Plasmid pUC18/neo(Bam) DNA is digested with endonucleases SacII and HindIII and the 4.0 kbp vector fragment is isolated by agarose gel electrophoresis. The two fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid pCIB4 (Fig. 4) contains the T-DNA right border and the plant-selectable marker for kanamycin resistance in a derivative of plasmid pUC18.

Next, a plasmid is constructed which contains both the T-DNA left and right borders, with the plant selectable kanamycin-resistance gene and the polylinker of pUC18 between the borders (Fig. 5). Plasmid pCIB4 DNA is digested with endonuclease HindIII, followed by treatment with the large fragment of DNA polymerase to create flush ends, followed by digestion with endonuclease EcoRI. The 2.6 kbp fragment containing the chimeric kanamycin resistance gene and the right border of T-DNA is isolated by agarose gel

electrophoresis. Plasmid pCIB5 DNA is digested with endonuclease AatII, treated with T4 DNA polymerase to create flush ends, then cleaved with endonuclease EcoRI. The larger vector fragment is purified by agarose gel electrophoresis, mixed with the pCIB4 fragment, incubated with T4 DNA ligase, and transformed into *E. coli* strain HB101. The resulting plasmid pCIB2 (Fig. 5) is a derivative of plasmid pBR322 containing the desired sequences between the two T-DNA borders.

The following steps complete the construction of the vector pCIB10 (Fig. 6). Plasmid pCIB2 DNA is digested with endonuclease EcoRV, and synthetic linkers containing BglII recognition sites are added as described above. After digestion with an excess of BglII endonuclease, the approximately 2.6 kbp fragment is isolated after agarose gel electrophoresis. Plasmid pRK252/Tn903/BglII (Fig. 1) is digested with endonuclease BglII and then treated with phosphatase to prevent recircularization. These two DNA fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid is the completed vector, pCIB10.

Plasmid pCIB10 comprises T-DNA borders containing a plant expressible NPT gene and convenient restriction endonuclease sites for insertion of other chimeric genes within the T DNA borders.

Example 10: Synthesis of Genes Encoding Insect-Selective Toxins

A. Purification of Insect Selective Toxins. The purification of several insect toxins has already been described in several works by Zlotkin (Zlotkin et al., 1971a and 1985; Lester et al., 1982). An alternative method which allows purification with high recoveries from limited amounts of venom is to use HPLC. This technique is exemplified in the purification of LqhIT2.

For purification of LqhIT2, lyophilized *L. quinquestriatus hebraeus* venom (Sigma) is extracted three times using 0.5 ml water/20 mg venom. The water extracts are combined and subjected to ion-exchange chromatography on sulfoethylaspartamide HPLC column (Nest Group). The extract is applied to the column previously equilibrated in 5 mM KPO_4 pH 3.0 in 25 % acetonitrile and the column eluted with a gradient from 0 to 0.5 M KCl in the same buffer over 60 min. Individual fractions are desalted and further separated by reverse phase chromatography on a Vydac C-8 column equilibrated in 0.1 % TFA and eluted with a 75 minute gradient from 0 to 70 % B (B: acetonitrile : isopropanol = 1:1 in

0.1 % TFA). Individual fractions are tested for toxicity to insects by injection into *Sarcophaga* and *Heliothis* larvae as described by Zlotkin et al. (1985).

B. Amino Acid Sequencing of Insect Toxins. The insect toxin is reduced by incubating samples in 6 M guanidine HCl, 1 M Tris HCl, pH 8.6, 10 mM EDTA, 20 mM dithiothreitol for 1 h at 37°C. 4-Vinylpyridine (Sigma) is added to 50 mM and incubation continued at room temperature for 1 h. The modified protein is desalted on a Vydac C-8 column as described above. Peptides are produced by enzymatic digestion with trypsin, Lys-C, or Glu-C or by partial acid hydrolysis following standard procedures (Allen, 1981). Peptides are separated by reverse phase HPLC prior to sequencing. The amino acid sequences of the intact toxin and the individual peptides are determined by automatic Edman degradation using a Model 470A Protein Sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line reverse phase HPLC for analysis of the phenylthiohydantoin derivatives of the amino acids and a Model 900 data analysis system.

Sequences of other insect toxins determined using the same techniques are provided in Fig. 7.

C. Synthesis of Gene Encoding Insect Toxin. Since the insect toxins are small proteins (< 80 amino acids), a gene encoding a toxin may be constructed by DNA synthesis. The following describes the synthesis of a gene which encodes AaIT, the *Androctonus australis* insect toxin.

The published sequence (Darbon et al., 1982) is back-translated using the genetic code with the codon frequency calculated from all available corn proteins in the GenBank data bank using the computer programs of the University of Wisconsin Genetics Computer Group. In some cases alternative codons may be selected to facilitate synthesis and/or provide convenient restriction sites. Translational stop and start signals are added along with BamHI linkers at both ends for convenience in subsequent manipulations. This process yields Sequence 1a and Sequence 1b (Fig. 8).

Oligonucleotides corresponding to the regions 1 to 20 (Sequences 1c) are synthesized using a Model 380A DNA synthesizer (Applied Biosystems, Foster City, CA) with β-cyanoethyl chemistry.

The gene is assembled in the following steps:

1) Set up the following reaction mixtures containing 40 pMol of the indicated fragments.

A. Fragments 2, 12, 13
B. Fragments 3, 4, 14, 15
C. Fragments 5, 6, 7, 16, 17, 18
D. Fragments 8, 9, 19, 20
E. Fragments 10, 11, 21

2) A 5' phosphate is added to the 5' ends of the fragments in each mixture by using T4 polynucleotide kinase according to the method described by Maniatis et al. (1982).

3) After removal of excess reagents by phenol/chloroform extraction, chloroform extraction, and ethanol precipitation, the precipitate of each mixture which contains the phosphorylated fragments is dissolved in T4 ligase buffer. 40 pMol of fragment 1 is added to mixture A and 40 pMol of fragment 22 is added to mixture E. The mixtures are heated to 85°C, then slow-cooled to 15°C and maintained at 15°C for at least 4 h to allow fragments to anneal.

4) ATP is added to 1 mM along with T4 ligase and incubation is continued for 4 h. Reagents are removed by extraction and precipitation as in step 1. To check for the efficacy of the reaction, an aliquot of the product is analyzed on a 10 to 15 % acrylamide gel. If necessary, the desired fragment is purified from each reaction mixture by preparative gel electrophoresis and recovered from the gel. Contaminates are again removed by precipitation.

The following fragment sizes are expected from the first set of ligations:

Mixture A: 49 bp
Mixture B: 45 bp
Mixture C: 65 bp
Mixture D: 45 bp
Mixture E: 46 bp

5) The products from the first ligations of A and B are mixed in reaction F. Products from ligations D and E are mixed for reaction G. Steps 3) and 4) are repeated on mixtures F and G. This results in a 89 bp fragment from reaction F and an 86 bp fragment from reaction G.

6) The purified fragments from F, G and C are mixed together and steps 3) and 4) repeated to give the final gene of 230 bp with BamHI ends. The final sequence is shown in sequence 1d (Fig. 8). This purified fragment is used for ligation into the BamHI site of appropriate vectors.

7) To amplify the DNA, the purified fragment is ligated into the BamHI site of pUC18 and cloned in a suitable *E. coli* host. The DNA sequence of the insert is confirmed using standard sequencing methodology.

Example 11: Plant transformation vector comprising CaMV 35S promoter/toxin chimeric genes

Vectors containing a plant expressible promoter with sites for inserting heterologous coding sequences are derived from pCIB10 (Rothstein et al., 1987). The plasmid pCIB770 contains the 35S promoter from CaMV as the plant expressible promoter. The coding sequence for a toxin having insecticidal activity, is ligated downstream of the promoter using BamHI cloning sites.

Plant tissue transformed with this vector is selected using the antibiotics kanamycin or G418 as detailed below and as known to those skilled in the art.

Example 12: Plant transformation vectors comprising chimeric plant expressible toxin genes and a hygromycin resistance marker for plant selection

The plasmid pCIB743 (Rothstein et al., 1987) contains a plant expressible hygromycin resistance gene within the T DNA borders. A second plant expressible chimeric gene is inserted using the unique restriction sites and this is introduced into plants.

Plant tissue transformed with this vector is selected using the antibiotic hygromycin or an analogous antiobiotic as detailed below and as known to those skilled in the art.

Example 13: Leaf Disk Transformation of Tobacco

A: *Agrobacterium* Infection of Plant Material. The different genotypes of *A. tumefaciens* are grown on AB minimal medium (Watson et al., 1975) plus mannitol or glutamate salts medium for 48 hr at 28°C. Bacteria are pelleted, resuspended in MSBN medium at a two-fold dilution, and held for three hours at 25°C. MSBN medium is comprised of full-strength major and minor salts of Murashige and Skoog (Murashige and Skoog, 1962) (KC Biologicals) with the following additions (final concentrations): 6-benzyladenine (1 mg/l); nicotinic acid (1 mg/l); pyridoxine (1 mg/l); thiamine HCl (10 mg/l); and sucrose (30 g/l). The pH is adjusted to 5.8. 5 to 7 mm leaf disks are punched aseptically from in vitro cultured *Nicotiana tabacum* cv. Xanthi plants and are dipped into the bacterial suspension for 10 minutes in a modification of the method of Horsch et al. (1985). Leaf disks are then transferred to filter paper on MSBN medium. After 48 hr, the leaf disks are dipped in liquid MSBN medium containing 500 mg/l of carbenicillin and transferred to solid (0.8 % agar) MSBN medium containing 100 mg/l kanamycin and 500 mg/l carbenicillin for selection of transformed cells.

B. Plant Maturation and Self-Pollination. Shoots that arose from calli on MSBN selection medium are transferred to OMS medium which is comprised of MS major, minor salts and Fe-EDTA (Gibco # 500-1117; 4.3 g/l), B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose, pH 5.8) and supplemented with 100 mg/l kanamycin and 250 mg/l carbenicillin. Kanamycin and carbenicillin are added as a filter sterilized solution after autoclaving the rest of the medium. Plantlet development is allowed to continue for at least three weeks. Plantlets are divided to give replicate cuttings which are allowed to develop and root for at least three weeks. Rooted plantlets are then transplanted to a soil-vermiculite mixture and moved to the greenhouse. Freshly potted plantlets are kept moist and shaded under an inverted clear plastic beaker for hardening off. At flowering time flowers are induced to self pollinate. Seeds are harvested following maturation.

Example 14: Production of Transgenic Tobacco Callus and Plants

The T DNA containing vector or the vector comprising the chimeric genes expressible in plants is transferred from *E. coli* SM17 (Simon et al., 1983) to *A. tumefaciens* strain CIB542 by mating. Alternatively, the vector is transformed into *Agrobacterium* strain CIB542 by the method of Holsters et al. (1978). *Agrobacterium* strain CIB542 is strain

EHA101 (Hood et al., 1986) in which the kanamycin marker of the plasmid has been replaced by the spectinomycin/streptomycin portion of Tn7. *Agrobacterium* strains bearing the T DNA derived plasmid and the CIB542 are used to transform tobacco by the leaf disk method as described above. Kanamycin-resistant transformed plants are grown to maturity. Alternatively, callus forming from the leaf disks on kanamycin-containing MSBN selection medium is maintained on a callus growth medium comprised of MS major, minor salts and Fe-EDTA (Gibco # 500-1117; 4.3 g/l), MS vitamins, 100 mg/l myo-inositol, 20 g/l sucrose, 2 mg/l naphthaleneacetic acid and 0.3 mg/l kinetin.

The callus can be used to regenerate transgenic plants by transferring callus pieces to MSBN medium and following methods as described above.

Example 15: Transformation and Regeneration of *Zea mays*

Zea mays is transformed and regenerated in the media set forth in Table 9.

Table 9: Composition of Media Used

Macroelements, microelements and Fe-EDTA of the media are as given in the literature: KM medium according to Kao and Michayluk (1975); N6 medium according to Chu et al. (1975).

Medium	KM-8p	N6
Organics and Vitamins Used in the Culture Media: [mg/l]		
Biotin	0.01	
Pyridoxine HCl	1.00	0.5
Thiamine HCl	10.00	0.1
Nicotinamide	1.00	
Nicotinic acid	0.10	0.5
Folic Acid	0.40	
D-Ca-Pantothenate	1.00	
p-Aminobenzoic Acid	0.02	
Choline Chloride	1.00	
Riboflavin	0.20	

Vitamin B-12	0.20	
Glycine	0.10	2.0
Sugars and sugar alcohols: [g/l]		
Sucrose	0.25	30.0
Glucose	68.40	
Mannitol	0.25	
Sorbitol	0.25	
Cellobiose	0.25	
Fructose	0.25	
Mannose	0.25	
Ribose	0.25	
Xylose	0.25	
Myo-Inositol	0.10	
Final pH	5.8	5.6
Sterilization	filter	autoclaved

Macroelements are usually made up as a 10 x concentrated stock solution, and microelements as a 1000 x concentrated stock solution.

Citric, fumaric and malic acid (each 40 mg/liter final concentration) and sodium pyruvate (20 mg/liter final concentration) are prepared as a 100 x concentrated stock solution, adjusted to pH 6.5 with NH_4OH, and added to this medium.

Adenine (0.1 mg/liter final concentration), and guanine, thymidine, uracil, hypoxanthine and cytosine (each 0.03 mg/liter final concentration) are prepared as a 1000 x concentrated stock solution, adjusted to pH 6.5 with NH_4OH, and added to this medium.

The following amino acids are added to this medium using a 10 x stock solution (pH 6.5 with NH_4OH) to yield the given final concentrations: glutamine (5.6 mg/liter), alanine, glutamic acid (each 0.6 mg/liter), cysteine (0.2 mg/liter) asparagine, aspartic acid, cystine, histidine, isoleucine, leucine,

lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine (each 0.1 mg/liter).

Vitamin stock solution is normally prepared 100x concentrated.

Example 16: Preparation of Antibodies to Insect-Selective Toxins

A. Preparation of Immunogen. Standard computer analyses (Hopp and Woods, 1983) are used to predict antigenic sites from the amino acid sequences of insect selective toxins. Synthetic peptides are prepared corresponding to these regions. The peptides are coupled through an interval cysteine to an ovalbumin carrier using the reagent N-succinimidyl-3-(2-pyridyldithio)propionate (Pierce Chemical Co.) (Carlsson et al., 1978). The degree of conjugation is estimated by amino acid analysis of the conjugate.

B. Production of Antisera. Rabbits are immunized with 0.5 to 1.0 mg antigen emulsified in complete Freund's adjuvant and boosted monthly with antigen in incomplete Freund's adjuvant. Sera are titered by conventional ELISA assays using the peptide conjugated to a heterologous carrier (typically bovine serum albumin). Positive sera are titered against the appropriate insect toxin.

C. Results. Typically dilutions of 1:10,000 allow detection of 1 to 10 ng of the homologous peptides. Dilutions of 1:300 allow detection of 3 to 10 ng of intact toxin protein. Table 10 shows results of immunization with several different toxin peptides.

Table 10: Detection Limits in ELISA Assay of Antisera Raised against Toxin Peptides

Toxin	Immunizing Peptide	Detection of Peptide	Detection of Toxin
AaIT	N-terminal 1-16	3 ng at 1:10,000	3 ng at 1:300
AaIT	C-terminal 52-70	3 ng at 1:10,000	3 ng at 1:300
LqhIT2	N-terminal 1-13	1 ng at 1:1,000	
LqhIT2	C-terminal 46-61	1 ng at 1:10,000	
BjIT2	N-terminal 1-13	0,3 ng at 1:3,000	
BjIT2	C-terminal 46-60	10 ng at 1:3,000	1 ng at 1:300

Example 17: Maize Resistant to Corn Root Worm by Expression of AaIT

A. Preperation of vector. The synthetic AaIT gene prepared as described in Example 9 is ligated into the BamHI site of pCIB710 (Rothstein et al., 1987) as described above. A gene for the desired selective marker (e.g., the NPT gene conferring kanamycin resistance) is ligated into one of the multiple cloning sites using standard techniques.

B. Transformation and Regeneration of Maize. Maize tissue is transformed with the pCIB710 vector carrying the AaIT gene insert and plants are regenerated as described above. For controls, plants transformed with the pCIB710 vector alone are prepared in the same way. The initial plants are self-fertilized and seed (T1 seed) is obtained.

C. Testing Plants for AaIT Expression. Plants grown from the T1 seed are analyzed for the presence and expression of the AaIT gene using several tests.

1) DNA is isolated and digested with BamHI; the digest is electrophoresed on a 1.5 % agarose gel. The DNA fragments are transferred to nitrocellulose and hybridized with the AaIT gene labeled with ^{32}P by nick translation (Maniatis et al., 1982). The presence of the AaIT gene is detected by a band of approximately 230 bp which hybridizes to the probe.

2) RNA is detected by the Northern blot procedure (Maniatis et al., 1982) as a band of approximately 230 bases which hybridizes with the ^{32}P-AaIT gene described above.

3) AaIT protein is detected using standard immunological techniques with polyclonal rabbit antibodies raised against synthetic peptides corresponding to the N-terminal 16 amino acids of AaIT and the C-terminal 19 amino acids of AaIT (see Example 16).

4) AaIT activity is detected by immunopurifying material from the plant extracts using the rabbit polyclonal anti-AaIT antibody and protein A Sepharose and assaying the isolated material for toxicity to insects by injecting the material into *Sarcophaga* larvae using the methods described in Example 10.

D. Resistance of Transformed Corn Plants to Corn Root Worm Damage. Germinated T1 seeds are planted in coarse vermiculite in 100 mm Petri dishes (5/dish, 5 dishes). When the second leaves on the seedling emerge, each dish is infested with 20 second instar corn root worm larvae. After seven days, the number and weight of the survivors is measured along with the weight of the washed roots of the corn plant. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root weight relative to insect-free plants when plants expressing the AaIT gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 18: Potato and Tomato Resistant to Colorado Potato Beetle by Expression of AaIT

A. Construction of Vector. The AaIT gene is synthesized and ligated into vector pCIB710 as described in Example 17, section A. The AaIT gene plus the 35S CaMV promoter are subcloned out of the pCIB710 vector into the pCIB10 vector by digesting with XbaI and EcoRI, isolating the 1460 bp fragment and ligating this fragment with XbaI, EcoRI digested pCIB10 to make the pCIB10-AaIT vector.

B. Transformation and Regeneration of Plants. The pCIB10-AaIT vector is introduced into *A. tumefaciens* carrying a virulence plasmid such as LBA 4404 or pCIB542. pCIB542 is an *A. tumefaciens* plasmid bearing an engineered *vir* plasmid derived from pTiBo542 (Hood et al., 1986). pCIB542 has the bacterial kanamycin resistance gene replaced by a bacterial streptomycin/spectinomycin resistance gene. The strain carrying both pCIB710-AaIT and pCIB542 is used to produce transformed tomato plants according to the method of Fischhoff et al. (1987).

Potato plants containing pCIB10-AaIT are obtained by the method of Stockhaus et al. (1987).

C. Testing of Transformants for AaIT Expression. Testing of transformants for AaIT expression is carried out as described in Example 17, section C.

D. Resistance of Transformed Plants to Colorado Potato Beetle. Ten four-week-old plants are each infested with five second instar Colorado potato beetle larvae. Larvae are allowed to feed for four days at which time insect mortality, insect weight gain and amount of

damage to the plant are scored. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in plant damage when plants expressing the AalT gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 19: Orchard Grass Resistant to Coleopterans by Expression of AalT

A. Construction of Vector. The AalT gene is synthesized and ligated into vector pCIB710 along with the kanamycin resistance gene as described in Example 17, section A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described above.

C. Testing of Transformants for AalT Expression. Testing of transformants for AalT expression is carried out as described in Example 17, section C.

D. Resistance of Transformed Plants to Damage by *Diabrotica undecimpunctata* (Southern Corn Root Worm). Germinated T1 seeds are planted in fine soil in 100 mm Petri dishes (10/dish, 5 dishes). When the second leaves on the seedling emerge, each of five dishes is infested with 20 second instar *D. undecimpunctata* larvae. After seven days, the number and weight of the survivors is measured along with the weight of the washed roots of the corn plant. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root mass relative to insect-free plants when plants expressing the AalT gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 20: Cotton Resistant to Boll Weevil by Expression of AalT

A. Construction of Vector. The AalT gene in pCIB10 is prepared as described in Example 18, section A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described above.

C. Testing of Transformants for AaIT Expression. Testing of transformants for AaIT expression is carried out as described in Example 17, section C.

D. Resistance of Transformed Plants to Damage by *Anthonomus grandis* (Boll Weevil). Ten transformed plants are grown until bolls start to form. Each plant is infested with three adult female boll weevils. Damage to plants is rated after one week and surviving adults are removed. Damage by larva, larval numbers and weight per plant are measured at weekly intervals for four weeks. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in damage ratings, decrease in larval number, or decrease in larval weight when plants expressing the AaIT gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 21: Maize Resistant to Lepidopterous Pests by Expression of LqhIT2

A. Construction of pCIB710-LqhIT2 Vector. A synthetic gene for LqhIT2 is prepared using the general strategy outlined for the AaIT gene in Example 9. The resulting gene has the sequence shown in Sequence 2 (Fig. 9).

The resulting 200 bp fragment is isolated and ligated into the BamHI site of pCIB710 vector as described in Example 17, section A. A gene for the desired selective marker (e.g., the NPT gene conferring kanamycin resistance) is ligated into one of the multiple cloning sites using standard techniques. The resulting vector is denoted pCIB710-LqhIT2.

B. Transformation and Regeneration of Corn. Transformation and regeneration of corn plants is performed as described in Example 17, section B.

C. Testing Plants for LqhIT2 Expression. Plants grown from the T1 seed are analyzed for the presence and expression of the LqhIT2 gene using several tests.

1) DNA is isolated and digested with BamHI; the digest is electrophoresed on a 1.5 % agarose gel. The DNA fragments are transferred to nitrocellulose and hybridized with the LqhIT2 gene labeled with ^{32}P by nick translation (Maniatis et al. 1982). The presence of the LqhIT2 gene is detected by a band of approximately 200 bp which hybridizes to the probe.

2) RNA is detected by the Northern blot procedure (Maniatis et al., 1982) as a band of approximately 200 bases which hybridizes with the ^{32}P-LqhIT2 gene described above.

3) LqhIT2 protein is detected using standard immunological techniques with polyclonal rabbit antibodies raised against synthetic N- and C-terminal peptides of LqhIT2 as described in Example 16.

4) LqhIT2 activity is detected by immunopurifying material from the plant extracts using the rabbit polyclonal anti-LqhIT2 antibody and protein A Sepharose and assaying the isolated material for insect toxicity by injecting into *Heliothis* larvae as described in Example 10.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seeds are germinated and leaf pieces obtained from seedlings at the four leaf stage are used to feed neonatal European corn borer (*Ostrinia nubilalis*) or corn earworm (*Heliothis zea*) larvae. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After five days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from expressing the LqhIT2 gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 22: Cotton Resistant to Lepidopterous Insects by Expression of LqhIT2

A. Construction of Vector. The LqhIT2 gene together with the 35S CaMV promoter is removed from pCIB710 and ligated into pCIB10 using appropriate restriction enzymes. This vector is denoted as pCIB10-LqhIT2.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described above.

C. Testing Plants for LqhIT2 Expression. Testing of transformants for LqhIT2 expression is carried out as described in Example 21, section C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. Leaf disks from four-week-old transformed plants are fed to neonatal *Heliothis virescens*, *Heliothis zea* or *Pectinophora gossypiella*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days, insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from expressing the LqhIT2 gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 23: Tomato Resistant to Lepidopterous Larvae by Expression of LqhIT2

A. Construction of Vector. The pCIB10-LqhIT2 vector is prepared as described in Example 22, section A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 17, section B using the pCIB10-LqhIT2 vector in place of the pCIB10-AaIT vector.

C. Testing of Transformants for LqhIT2 Expression. Testing of transformants for LqhIT2 expression is carried out as described in Example 21, section C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Insects. Leaf disks from four-week-old transformed plants are fed to neonatal *Heliothis zea* or *Manduca sexta*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days, insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from expressing the LqhIT2 gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 24: Tobacco Resistant to Lepidopterous Larvae by Expression of LqhIT2

A. Construction of Vector. The pCIB10-LqhIT2 vector is prepared as described in Example 22, section A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described above using the pCIB10-LqhIT2 vector.

C. Testing of Transformants for LqhIT2 Expression. Testing of transformants for LqhIT2 expression is carried out as described in Example 21, section C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Insects. Leaf disks from four-week-old transformed plants are fed to neonatal *Heliothis virescens* (tobacco budworm) or *Manduca sexta* (tomato/tobacco hornworm). Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days, insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from expressing the LqhIT2 gene are compared to control plants transformed with the vector alone or untransformed plants.

Example 25: Orchard Grass Resistant to Damage by Lepidopterous Larvae through Expression of LqhIT2

A. Construction of Vector. The LqhIT2 gene is synthesized and ligated into vector pCIB710 as described in Example 21, section A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described above.

C. Testing of Transformants for LqhIT2 Expression. Testing of transformants for LqhIT2 expression is carried out as described in Example 21, section C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Insects. Germinated T1 seeds are planted in fine soil in 100 mm Petri dishes (10/dish, 5 dishes). When the second leaves on the seedling emerge, each dish is infested with 20 second instar larvae of corn root webworm (*Crambus caliginosellus*). After seven days, the number and weight of the survivors is measured along with the weight of the washed grass roots. Resistance of transformed plants is detected by a statistically significant (Student's t-test, $p < 0.05$)

decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root weight relative to insect-free plants when plants expressing the LqhIT2 gene are compared to control plants transformed with the vector alone or untransformed plants.

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~~WHAT IS CLAIMED IS:~~

The claims defining the invention are as foll.

1. An insect selective toxin comprising the following amino acid sequence:
VRDAYIAKNY NCVYECFRDA YCNELCTKNG A^SGYCQWAG KYGNACWCYA LPDNVPIRVP GKCR.

2. A recombinant DNA comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

3. A recombinant DNA according to claim 2 wherein said DNA sequence obtainable from animals is in an expressible form.

4. A recombinant DNA according to claim 2 or 3 wherein said DNA sequence encodes an insect selective toxin obtainable from arthropods.

5. A recombinant DNA according to claim 2 or 3 wherein said DNA sequence encodes an insect selective toxin obtainable from members of the classes *Arachnida* or *Chilopoda*.

6. A recombinant DNA according to claim 2 or 3 wherein said DNA sequence encodes an insect selective toxin obtainable from members of the order *Scorpiones*.

7. A recombinant DNA according to claim 2 or 3 wherein said DNA sequence encodes an insect selective toxin obtainable from members of the genus *Scolopendra*.

8. A recombinant DNA according to claim 2 or 3 wherein said DNA sequence encodes an insect selective toxin with the following amino acid sequence:
KKNGYAVDSS GKAPECLLSN YCNNQCTKVH YADKGYCCLL SCYCFGLNDD KKVLEISDTR KSYCDTTIIN,
DGYIRKRDGC KLSCLFGNEG CNKECKSYGG SYGYCWTWGL ACWCEGLPDE KTWKSETNTC G,
DGYIRKKDGC KVSC(V/I)IIGNEG CRKECVAHGG SFGYCWTWGL ACWCENLPDA VTWKSSTNTC G,
DGYIKRRDGC KVACLIGNEG CDKECKAYGG SYGYCWTWGL ACWCEGLPDD KTWKSETNTC G,

ALPLSGEYEP CVRPRKCKPG LVCNKQQICV DPK or
VRDAYIAKNY NCVYECFRDA YCNELCTKNG ASSGYCQWAG KYGNACWCYA
LPDNVPIRVP GKCR or a functional derivative or fragment thereof.

9. A vector comprising a DNA according to any one of claims 2 to 8.

10. A host organism comprising a vector according to claim 9.

11. A transgenic plant cell comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

12. A transgenic plant cell according to claim 11 wherein said DNA sequence is obtainable from arthropods.

13. A transgenic plant cell according to claim 11 wherein said DNA sequence is obtainable from members of the classes *Arachnida* or *Chilopoda*.

14. A transgenic plant cell according to claim 11 wherein said DNA sequence is obtainable from members of the order *Scorpiones*.

15. A transgenic plant cell according to claim 11 wherein said DNA sequence is obtainable from members of the genus *Scolopendra*.

16. A transgenic plant cell according to any one of claims 11 to 15 wherein said DNA sequence is stably integrated into the plant genome.

17. A transgenic plant cell according to claim 16 wherein said DNA sequence is in an expressible form.

18. A transgenic plant cell according to claim 17 that expresses an insect selective toxin encoded by said DNA sequence, or a functional derivative or fragment thereof.

19. A transgenic plant and its sexual and asexual progeny comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof.

20. A transgenic plant and its sexual and asexual progeny according to claim 19 wherein said DNA sequence is obtainable from arthropods.

21. A transgenic plant and its sexual and asexual progeny according to claim 19 wherein said DNA sequence is obtainable from members of the classes *Arachnida* or *Chilopoda*.

22. A transgenic plant and its sexual and asexual progeny according to claim 19 wherein said DNA sequence is obtainable from members of the order *Scorpiones*.

23. A transgenic plant and its sexual and asexual progeny according to claim 19 wherein said DNA sequence is obtainable from members of the genus *Scolopendra*.

24. A transgenic plant and its sexual and asexual progeny according to any one of claims 19 to 23 wherein said DNA sequence is stably incorporated in the plant genome.

25. A transgenic plant and its sexual and asexual progeny according to claim 24 wherein said DNA sequence is in an expressible form.

26. A transgenic plant and its sexual and asexual progeny according to claim 25 that expresses an insect selective toxin encoded by said DNA sequence, or a functional derivative or fragment thereof.

27. A transgenic microorganism comprising a DNA sequence obtainable from animals encoding an insect selective toxin or a functional derivative or fragment thereof.

28. A transgenic microorganism according to claim 27 wherein said DNA sequence is obtainable from arthropods.

29. A transgenic microorganism according to claim 27 wherein said DNA sequence is obtainable from members of the classes *Arachnida* or *Chilopoda*.

30. A transgenic microorganism according to claim 27 wherein said DNA sequence is obtainable from members of the order *Scorpiones*.

31. A transgenic microorganism according to claim 27 wherein said DNA sequence is obtainable from members of the genus *Scolopendra*.

32. A transgenic microorganism according to any one of claims 27 to 31 wherein said DNA sequence is stably incorporated in the genome.

33. A transgenic microorganism according to claim 32 wherein said DNA sequence is in an expressible form.

34. A transgenic microorganism according to claim 33 expressing the insect selective toxin encoded by said DNA sequence, or a functional derivative or fragment thereof.

35. An antibody for an insect selective toxin or a functional derivative or fragment thereof obtainable from members of the order *Scorpiones* or the genus *Scolopendra*.

36. An agricultural formulation comprising as active substance an insect selective toxin or a functional derivative or fragment thereof obtainable from animals and one or more agricultural carrier and one or more additional agent with the proviso that the toxin is not dissolved in ethanol plus water, acetone plus water or acetone plus dimethyl sulphoxide.

37. An agricultural formulation according to claim 36 wherein the toxin is a recombinant toxin.

38. A method of controlling phytopathogenic insects comprising the application to the insect or its environment of an insecticidally effective amount of a transgenic microorganism according to any one of claims 27 to 34 or of a formulation according to claims 36 or 37.

39. A method of protecting crop plants against phytopathogenic insects comprising the transformation of the crop plant with a recombinant DNA according to claim 2 and further comprising the expression in the plant of an insecticidally effective amount of said insect selective toxin or a functional derivative or fragment thereof.

40. An insect selective toxin substantially as hereinbefore described with reference to any one of the Examples.

41. A transgenic plant cell comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof substantially as hereinbefore described with reference to any one of Examples 13 to 15 or 17 to 25.

42. A transgenic plant and its sexual and asexual progeny

comprising a DNA sequence obtainable from animals wherein said DNA sequence encodes an insect selective toxin or a functional derivative or fragment thereof substantially as hereinbefore described with reference to any one of the Examples.

43. A transgenic microorganism comprising a DNA sequence obtainable from animals encoding an insect selective toxin or functional derivative or fragment thereof substantially as hereinbefore described with reference to any one of the Examples.

44. A recombinant DNA substantially as herein described with reference to any one of Examples 10 to 15 or 17 to 25.

45. A vector substantially as herein described with reference to any one of Examples 9, 11 to 15 or 17 to 25.

46. A host organism substantially as herein described with reference to any one of Examples 9, 11 to 15 or 17 to 25.

47. An antibody for an insect selective toxin or a functional derivative or fragment thereof substantially as herein described with reference to Example 16.

48. An agricultural formulation comprising as active substance an insect selective toxin as defined in claim 40 or a functional derivative or fragment thereof obtainable from animals and one or more agricultural carrier and one or more additional agent with the proviso that the toxin is not dissolved in ethanol plus water, acetone plus water, or acetone plus dimethyl sulphoxide.

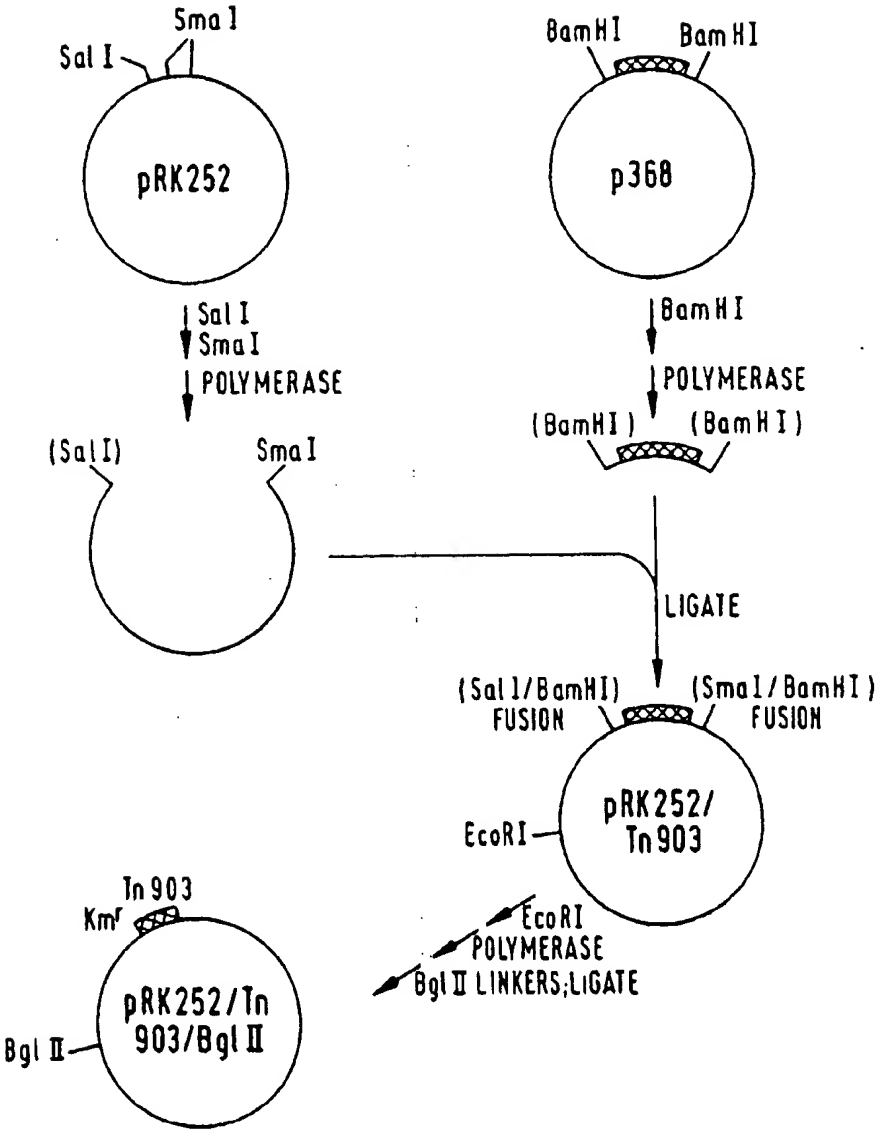
49. A method of controlling phytopathogenic insects comprising applying to said insect or its environment an insecticidally effective amount of a transgenic microorganism as defined in claim 43 or a formulation as defined in claim 48.

50. A method of protecting crop plants against phytopathogenic insects comprising transforming said crop plant with a recombinant DNA as defined in claim 44 and further comprising the expression in the plant of an insecticidally effective amount of said insect selective toxin or a functional derivative or fragment thereof.

DATED this SIXTH day of OCTOBER 1992
Ciba-Geigy AG

Patent Attorneys for the Applicant
SPRUSON & FERGUSON

Fig. 1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(BamHI)
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(Sal I/BamHI)
FUSION
(Sma I/BamHI)
FUSION
pRK252/
Tn 903
EcoRI
EcoRI
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Bgl II

Fig. 2

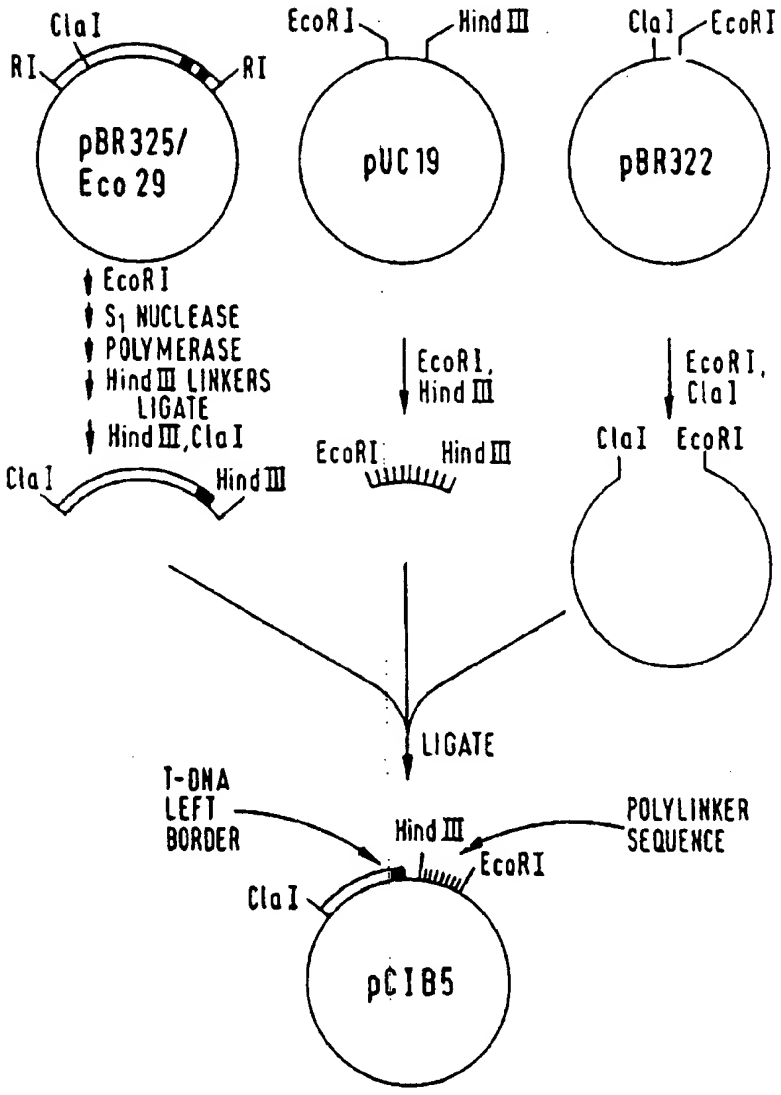
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Fig. 3

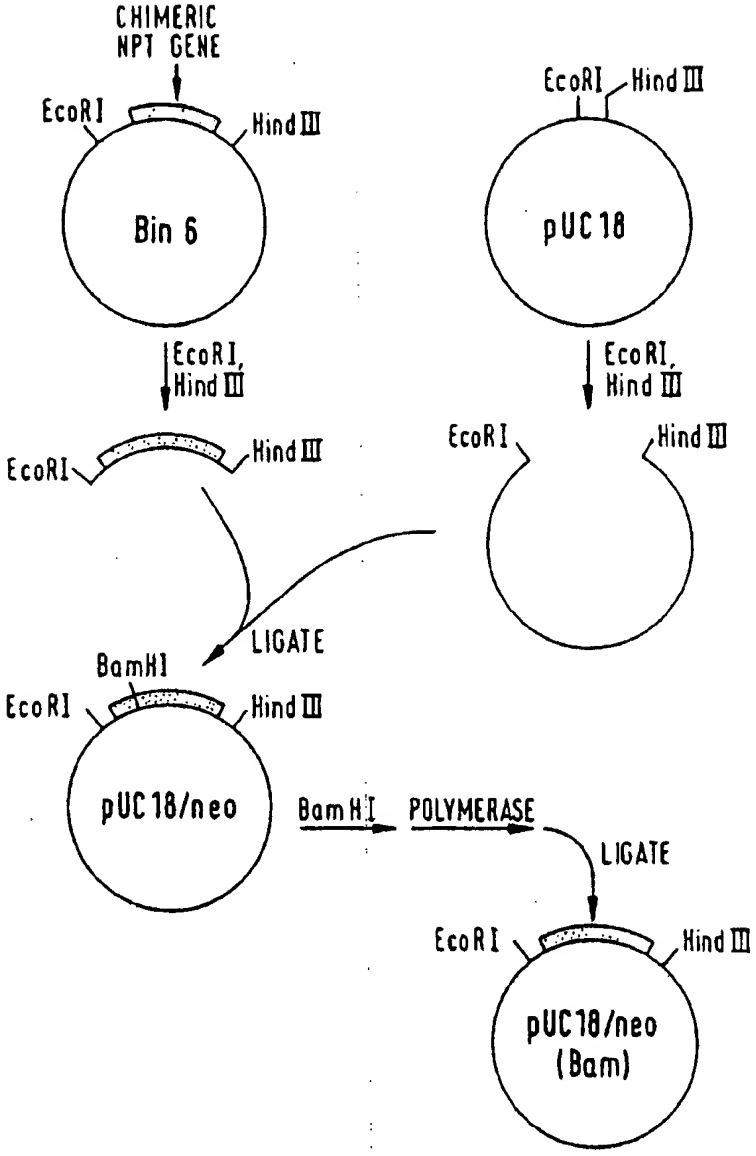
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Fig. 4

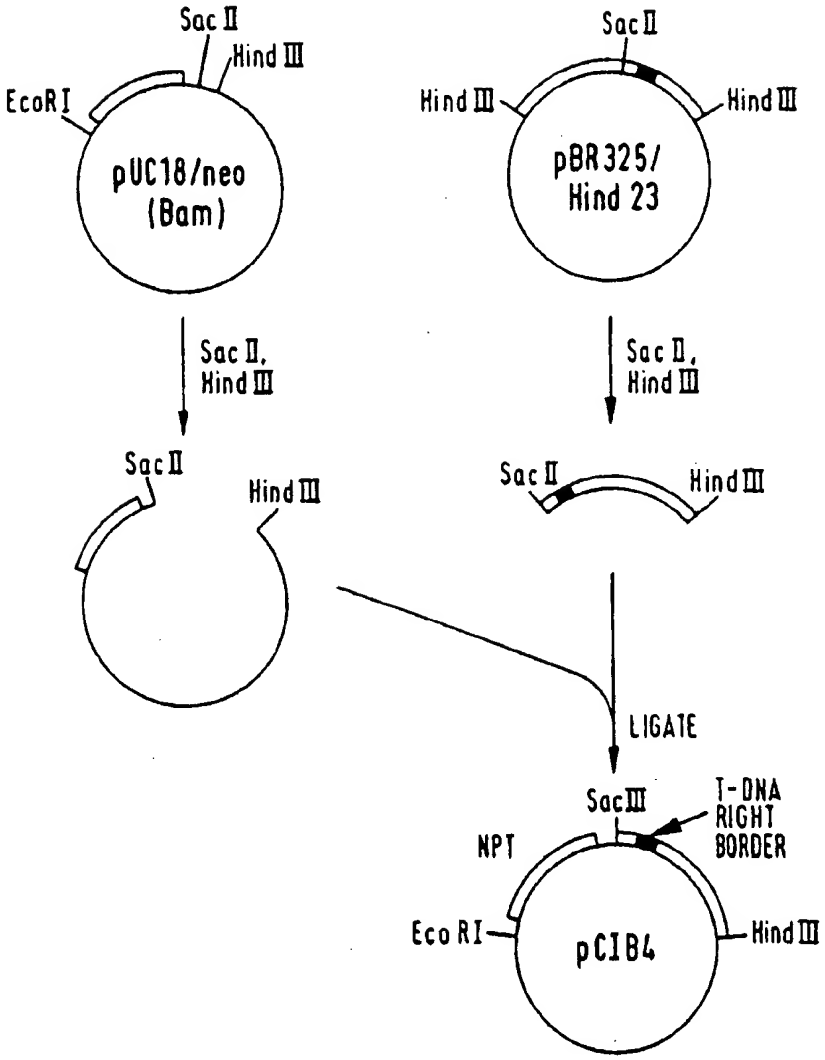
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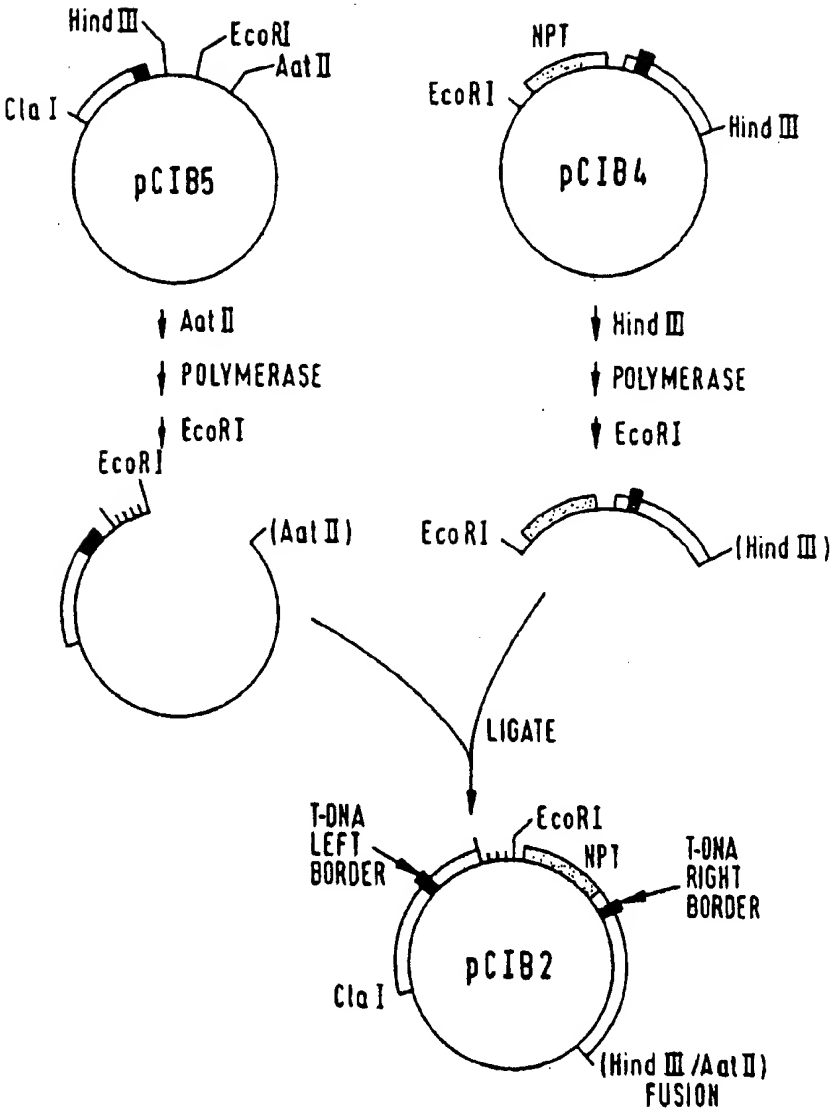
Fig. 5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Hind III
POLYMERASE
EcoRI
EcoRI
(Aat II)
EcoRI
(Hind III)
LIGATE
T-DNA
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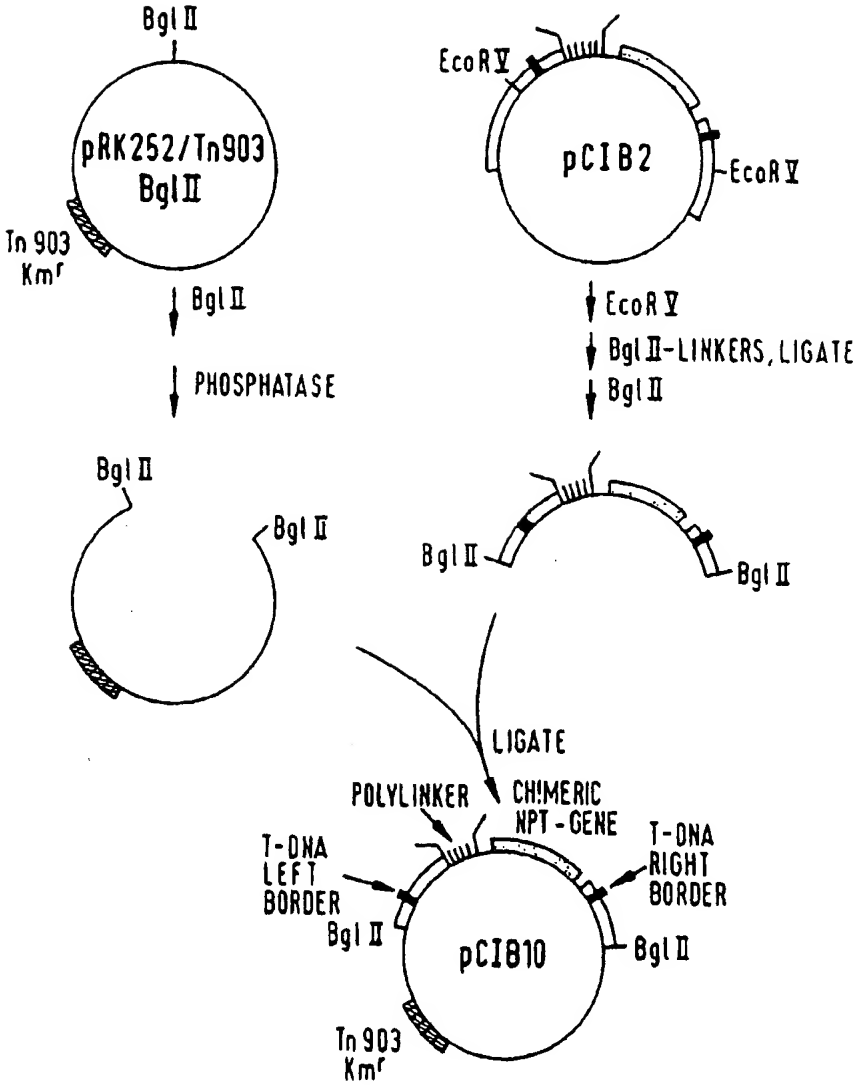
RIGHT
BORDER
pCIB2
Cla I
(Hind III /Aat II)
FUSION

Fig. 6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EcoR V
EcoR V
Bgl II-LINKERS, LIGATE
Bgl II
Bgl II
Bgl II
LIGATE
POLYLINKER
CHIMERIC
NPT-GENE
T-DNA
LEFT
BORDER
T-DNA
RIGHT
BORDER
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pCIB10
Bgl II
Tn 903
Km^r

Scorpion Toxin Sequences

Buthoid Scorpion Toxins

LqhIT2	DGYIKRRDGC	KVACLIGNEG	CDKECKAYGG	SYGYCWTWGL	ACWCEGLPDD	KTWKSETNTC	G
LqqIT2	DGYIRKRDGC	KLSCLFGNEG	CNKECKSYGG	SYGYCWTWGL	ACWCEGLPDE	KTWKSETNTC	G
BjIT2	DGYIRKKDGC	KVSCIIGNEG	CRKECVAHGG	SFGYCWTWGL	ACWCENLPDA	VTWKSSTNTC	G
LqhP35	VRDAYIAKNY	NCVYECFRDA	YCNELCTKNG	ASSGYCQWAG	KYGNACWCYA	LPDNVPIRVP	GKCR

Chaotid Scorpion Toxins

SmpIT2	ALPLSGEYEP	CVRPRKCKPG	LVCNKQQICV	DPK	
SmpCT2	VSCTGSRDCY	APCKRQTGCT	SAKCINKSCK	CYGC	
SmpCT3	VSCTGSKDCY	APCRKQTGCP	NAKCINKSCK	CYGC	
SmpMT	VSCTGSKECY	APCKKQTGCP	NAKCMNRKCK	CYGC	

Fig. 7

SYNTHESIS OF GENE FOR AAIT

SEQUENCE 1a
Coding strand

```
5'   1  GATCCAAATA ATGAAAAAAA ACGGCTACGC TGTTGACTCT TCTGGCAAAG
        |_____1_____| |_____2_____| |_____

  51   CTCCGGAATG CCTGCTGTCT AACTACTGCA ACAACCAGTG CACTAAAGTT
       _____3_____| |_____4_____| |_____5_____

 101   CATTACGCTG ACAAAGGCTA CTGCTGCCTG CTGTCTTGCT ACTGCTTCGG
       ___| |_____6_____| |_____7_____| |_____

 151   CCTGAACGAC GACAAAAAAG TTCTGGAAAT CTCTGACACT CGTAAATCTT
       ___8_____| |_____9_____| |_____10_____

 201   ACTGCGACAC TACTATCAAC TAATAG     3'
       ___| |_____11_____|
```

SEQUENCE 1b
Complementary strand

```
5'   1  GATCCTATTAGTTG ATAGTAGTGT CGCAGTAAGA TTTACGAGTG TCAGAGATTT
        |_____22_____| |_____21_____| |_____20__

  55   CCAGAACTTT TTTGTCGTCG TTCAGGCCGA AGCAGTAGCA AGACAGCAGG
       _____| |_____19_____| |_____18_____| |___

 105   CAGCAGTAGC CTTTGTCAGC GTAATGAACT TTAGTGCACT GGTTGTTGCA
       _____17_____| |_____16_____| |_____15___

 155   GTAGTTAGAC AGCAGGCATT CCGGAGCTTT GCCAGAAGAG TCAACAGCGT
       _____| |_____14_____| |_____13_____| |___

 201   AGCCGTTTTT TTTCATTATT TG     3'
       _____12_____|
```

Fig. 8A

SEQUENCE 1c
Fragments Synthesized

1	GATCCAAATAATGAAAAAAAACGG
2	CTACGCTGTTGACTCTTCTG
3	GCAAAGCTCCGGAATGCCTG
4	CTGTCTAACTACTGCAACAA
5	CCAGTGCACTAAAGTTCATT
6	ACGCTGACAAAGGCTACTGC
7	TGCCTGCTGTCTTGCTACTG
8	CTTCGGCCTGAACGACGACA
9	AAAAAGTTCTGGAAATCTCT
10	GACACTCGTAAATCTTACTG
11	CGACACTACTATCAACTAATAG
12	CGTAGCCGTTTTTTTTCATTATTTG
13	TTTGCCAGAAGAGTCAACAG
14	GACAGCAGGCATTCCGGAGC
15	ACTGGTTGTTGCAGTAGTTA
16	AGCGTAATGAACTTTAGTGC
17	AGGCAGCAGTAGCCTTTGTC
18	CGAAGCAGTAGCAAGACAGC
19	TTTTTTGTCGTCGTTCAGGC
20	GTGTCAGAGATTTCCAGAAC
21	TGTCGCAGTAAGATTTACGA
22	GATCCTATTAGTTGATAGTAG

Fig. 8B

SEQUENCE 1d
Final Gene

```
                10                  30                  50
1   GATCCAAATAATGAAAAAAAACGGCTACGCTGTTGACTCTTCTGGCAAAGCTCCGGAATG   60
    GTTTATTACTTTTTTTTGCCGATGCGACAACTGAGAAGACCGTTTCGAGGCCTTAC
          M   K   K   N   G   Y   A   V   D   S   S   G   K   A   P   E   C

                70                  90                  110
61  CCTGCTGTCTAACTACTGCAACAACCAGTGCACTAAAGTTCATTACGCTGACAAAGGCTA  120
    GGACGACAGATTGATGACGTTGTTGGTCACGTGATTTCAAGTAATGCGACTGTTTCCGAT
      L   L   S   N   Y   C   N   N   Q   C   T   K   V   H   Y   A   D   K   G   Y

                130                 150                 170
121 CTGCTGCCTGCTGTCTTGCTACTGCTTCGGCCTGAACGACGACAAAAAAGTTCTGGAAAT  180
    GACGACGGACGACAGAACGATGACGAAGCCGGACTTGCTGCTGTTTTTTCAAGACCTTTA
      C   C   L   L   S   C   Y   C   F   G   L   N   D   D   K   K   V   L   E   I

                190                 210
181 CTCTGACACTCGTAAATCTTACTGCGACACTACTATCAACTAATAG               230
    GAGACTGTGAGCATTTAGAATGACGCTGTGATGATAGTTGATTATCCTAG
      S   D   T   R   K   S   Y   C   D   T   T   I   N   ★   ★
```

Fig. 8C

SEQUENCE 2
Gene encoding LqhIT2 Insect Toxin

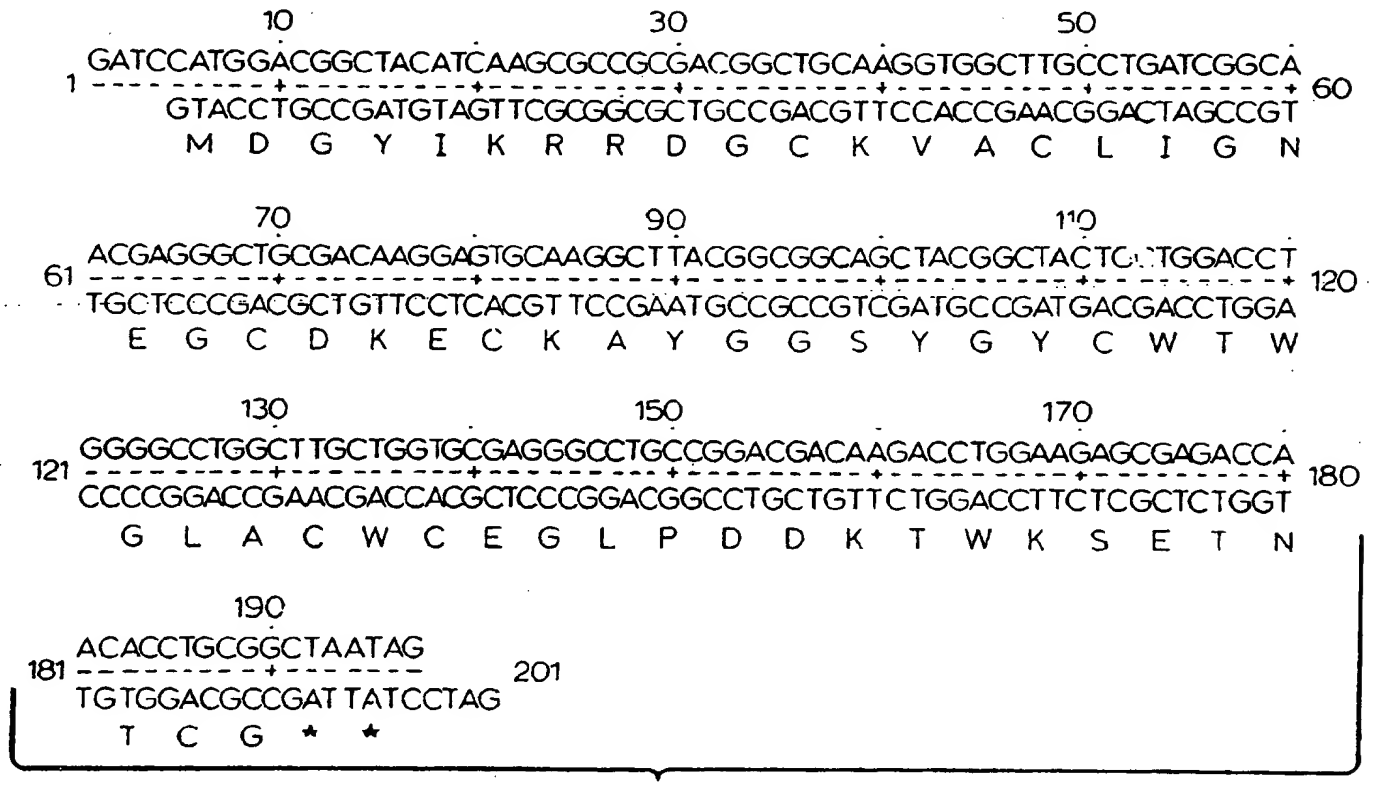

Fig. 9